

BLOOD PRESSURE LOWERING EFFECTS OF *LACTOBACILLUS*
HELVETICUS FERMENTED MILK CONTAINING BIOACTIVE
PEPTIDES ILE-PRO-PRO AND VAL-PRO-PRO:
MECHANISTIC, KINETIC AND CLINICAL STUDIES

Tiina Jauhiainen

Helsinki 2007

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Academic Dissertation

To be presented by kind permission of the Medical Faculty of the University of Helsinki
for public examination in Lecture Hall 2 of the Department of Internal Medicine,
Haartmaninkatu 4, on the 28th of September 2007, at 12 noon.

Helsinki 2007

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Layout: Oy Graaf Ab/Jani Osolanus

ISBN 978-952-92-2449-4 (paperback)

ISBN 978-952-10-4087-0 (PDF)

<http://ethesis.helsinki.fi>

Yliopistopaino
Helsinki 2007

CONTENTS

List of Original Publications	7
Abbreviations	8
Abstract	9
1 INTRODUCTION	11
2 REVIEW OF THE LITERATURE	13
2.1 Hypertension	13
2.1.1 Blood pressure and the definition of hypertension	13
2.1.2 Prevalence of hypertension	14
2.1.3 Hypertension and complications	15
2.1.4 Regulation of blood pressure	15
2.1.5 Treatment of hypertension	17
2.1.6 Blood pressure measurement	18
2.2 Renin-Angiotensin System	20
2.3 Vascular Endothelium	22
2.3.1 Endothelial dysfunction	23
2.3.2 Endothelium-derived vasodilatory factors	25
2.3.3 Endothelium-derived vasoconstrictory factors	26
2.4 Minerals and Blood Pressure	27
2.4.1 Sodium	27
2.4.2 Potassium	27
2.4.3 Calcium	28
2.4.4 Magnesium	29
2.5 Bioactive Peptides Derived from Milk Proteins	30
2.5.1 Peptides and blood pressure	31
2.5.2 Blood pressure lowering mechanisms	35
2.5.3 Peptides and vascular function	38
2.5.4 Kinetics of peptides	39
3 AIMS OF THE STUDY	41

4	MATERIALS AND METHODS	43
4.1	Experimental Animals and Study Designs.....	43
4.2	Subjects and Study Designs	44
4.3	Measurement of Blood Pressure.....	49
4.3.1	Tail-cuff method.....	49
4.3.2	Office blood pressure measurement.....	49
4.3.3	Home blood pressure measurement	49
4.3.4	Twenty-four-hour ambulatory blood pressure measurement.....	50
4.4	Arterial Responses	50
4.4.1	Arterial preparations and responses	50
4.4.2	ACE-inhibitory activity	50
4.4.3	Ambulatory arterial stiffness index	51
4.4.4	Pulse wave analysis	51
4.5	Collection of Samples	52
4.6	Biochemical Determinations	53
4.6.1	Peptide and mineral concentrations.....	53
4.6.2	Plasma and serum analyses	53
4.6.3	Urine analyses.....	53
4.6.4	Radioactivity analyses.....	53
4.6.5	Plasma protein binding properties	54
4.7	Products.....	54
4.8	Statistical Analysis.....	56
5	RESULTS	57
5.1	Effect of Peptide Milk on Blood Pressure	57
5.2	Effect of Peptide Milk on Vascular Function	60
5.3	Blood Pressure Lowering Mechanisms.....	62
5.4	Kinetics of Ile-Pro-Pro Peptide	63
6	DISCUSSION	65
6.1	Methodological Aspects.....	65
6.2	Effects of Peptide Milk on Blood Pressure and Vascular Function	69
6.3	Blood Pressure Lowering Mechanisms and Kinetics of Ile-Pro-Pro and Val-Pro-Pro	73
6.4	Clinical Relevance	75
7	SUMMARY AND CONCLUSIONS	77
8	ACKNOWLEDGEMENTS	79
9	REFERENCES	81

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (Studies I-VI) and some unpublished data.

- I **Jauhiainen T**, Collin M, Narva M, Poussa T, Cheng JZ, Poussa T, Vapaatalo H, Korpela R. Effect of long-term intake of milk peptides and minerals on blood pressure and arterial function in spontaneously hypertensive rats. *Milk Sci Int* 2005;60:358-63.

- II **Jauhiainen T***, Wuolle K*, Vapaatalo H, Kerojoki O, Nurmela K, Lowrie C, Korpela R. Oral absorption, tissue distribution and excretion of a radiolabelled analog of a milk-derived antihypertensive peptide, Ile-Pro-Pro, in rats. *Int Dairy J* 2007;17:1216-23. *equally contributed

- III Seppo L, **Jauhiainen T**, Poussa T, Korpela R. A fermented milk high in bioactive peptides has a blood pressure lowering effect in hypertensive subjects. *Am J Clin Nutr* 2003;77:326-30.

- IV **Jauhiainen T**, Vapaatalo H, Poussa T, Kyrönpalo S, Rasmussen M, Korpela R. *Lactobacillus helveticus* fermented milk lowers blood pressure in hypertensive subjects in 24-hour ambulatory blood pressure measurement. *Am J Hypertens* 2005;18:1600-5.

- V **Jauhiainen T**, Rönneck M, Vapaatalo H, Wuolle K, Kautiainen H, Korpela R. *Lactobacillus helveticus* fermented milk reduces arterial stiffness in hypertensive subjects. *Int Dairy J* 2007;17:1209-11.

- VI **Jauhiainen T***, Rönneck M*, Vapaatalo H, Wuolle K, Kautiainen H, Groop P-H, Korpela R. Long-term intervention with *Lactobacillus helveticus* fermented milk improves arterial stiffness in hypertensive subjects. Submitted 2007. *equally contributed

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ABBREVIATIONS

AASI	Ambulatory arterial stiffness index
ABPM	Ambulatory blood pressure measurement
ACE	Angiotensin-converting enzyme
AIx	Aortic augmentation index
Ang I	Angiotensin I
Ang II	Angiotensin II
AR	Adrenergic receptor
AT ₁	Angiotensin II type 1 receptor
AT ₂	Angiotensin II type 2 receptor
AUC	Area under the curve
BMI	Body mass index
cGMP	Cyclic guanosine -3',5'-monophosphate
CAGE	Chymostatin-sensitive angiotensin II-generating enzyme
COX	Cyclooxygenase
CRP	C-reactive protein
CVD	Cardiovascular disease
DASH	Dietary Approaches and Stop Hypertension
DBP	Diastolic blood pressure
dTGR	Double transgenic rats
EDHF	Endothelium-derived hyperpolarizing factor
ESC	European Society of Cardiology
ESH	European Society of Hypertension
ET	Endothelin
HPLC	High Performance Liquid Chromatography
Ile-Pro-Pro (IPP)	Isoleucyl-prolyl-proline
JNC	Joint National Committee
LC-MS	Liquid chromatography-mass spectrometry
NEP	Neutral endopeptidase
NHANES	National Health and Nutrition Examination Survey
NO	Nitric oxide
NOS	Nitric oxide synthase
PGI ₂	Prostacyclin
PRA	Plasma renin activity
PWA	Pulse wave analysis
RAS	Renin-angiotensin system
SBP	Systolic blood pressure
SD	Standard deviation
SHR	Spontaneously hypertensive rats
t-PA	Tissue plasminogen activator
Tr	Time to return of the reflected wave
TXA ₂	Thromboxane A ₂
Val-Pro-Pro (VPP)	Valyl-prolyl-proline
WHO-ISH	The World Health Organization and the International Society of Hypertension

ABSTRACT

The purpose of the present study was to evaluate the effects of *Lactobacillus helveticus* fermented milk (peptide milk) containing the casein-derived tripeptides Isoleucyl-prolyl-proline (Ile-Pro-Pro) and Valyl-prolyl-proline (Val-Pro-Pro) on blood pressure and vascular function in hypertensive subjects.

The peptide milk lowered systolic and diastolic blood pressure in long-term use in hypertensive subjects when blood pressure was measured by using 24-hour ambulatory blood pressure measurement (ABPM). The blood pressure lowering effect was seen with the dose of 50 mg of tripeptides, and a tendency for lowering blood pressure was also observed when the dose was 5 mg. No adverse effects compared to the placebo group were reported or detected in laboratory analysis.

The effect of the peptide milk on arterial stiffness was shown using two different methods, the ambulatory arterial stiffness index (AASI) and pulse wave analysis (PWA). According to the AASI, arterial stiffness was significantly reduced in the peptide milk group compared to the baseline level, but the difference was not significant compared to the placebo group. PWA showed that the peptide milk reduced arterial stiffness significantly compared to the placebo group. Endothelium-independent relaxation (nitroglycerin) and endothelium-dependent relaxation (salbutamol) did not differ between the groups.

The blood pressure lowering mechanisms of the tripeptides and the kinetics of Ile-Pro-Pro were investigated using spontaneously hypertensive rats (SHR) and Sprague-Dawley rats. Previous studies have suggested that the blood pressure lowering effect of the tripeptides Ile-Pro-Pro and Val-Pro-Pro is based on angiotensin-converting enzyme inhibition, but the present findings did not agree with these previous studies. It was shown in SHR that calcium, potassium and magnesium may also have an important role in attenuating the development of hypertension as part of the peptide milk effect. In addition, the present study suggests indirectly that improved endothelial nitric oxide release capacity is not the mechanism by which peptide milk mediates its favourable circulatory effects.

The kinetics of Ile-Pro-Pro were studied using adult Sprague-Dawley rats. The results showed that orally administered Ile-Pro-Pro is absorbed at least partly intact from the gastrointestinal tract. Radiolabelled Ile-Pro-Pro was distributed in different tissues and considerable radioactivity levels were found in tissues related to the renin-angiotensin system (RAS), adrenals, aorta and kidneys. Ile-Pro-Pro

does not bind to plasma proteins, and therefore it is possible that its blood pressure lowering effect is mediated by free Ile-Pro-Pro.

In conclusion, consumption of the peptide milk lowers blood pressure and reduces arterial stiffness in hypertensive subjects. Ile-Pro-Pro can be absorbed partly intact from the gastrointestinal tract and might accumulate in tissues related to the RAS. The precise blood pressure lowering mechanism of peptide milk remains to be studied.

1 INTRODUCTION

Hypertension is a major risk factor for cardiovascular diseases, including heart failure, coronary heart disease, peripheral artery disease and stroke. Hypertension is a worldwide health problem and can cause serious complications as well as significant costs to society. Expenditure on the prevention or early identification and subsequent treatment of hypertension, both with and without medication, are thus well worthwhile ways to reduce the overall risk of cardiovascular morbidity and mortality (Chobanian et al. 2003; Whitworth 2003; Mancia et al. 2007). Endothelial dysfunction is another risk factor for cardiovascular diseases, and it has been associated with cardiovascular risk factors such as hypertension and dyslipidaemia (Panza et al. 1990; Mensah et al. 2007; for reviews, see Vapaatalo and Mervaala 2001; Vanhoutte 2003). Evidence of an association between endothelial dysfunction and arterial stiffness has been demonstrated (Wilkinson et al. 2002b). Arterial stiffness is an independent predictor of cardiovascular morbidity and mortality (Dart et al. 1993; Boutouyrie et al. 2002).

Life-style factors, such as nutrition (for reviews, see Karppanen 1991; Nurminen et al. 1998; Myers and Champagne 2007), moderation of alcohol use (Xin et al. 2001), physical activity (Hagberg et al. 2000), weight reduction in those who are overweight (He et al. 2000) and abstinence from smoking (Doll et al. 2004) have a considerable impact on the treatment and prevention of hypertension and in lowering cardiovascular risks. Several studies have shown that a low intake of sodium (Cutler et al. 1997; Graudal et al. 1998; Kotchen and McCarron 1998; Sacks et al. 2001) and a sufficient intake of calcium (Allender et al. 1996; Bucher et al. 1996; van Mierlo et al. 2006), potassium (Cappuccio and MacGregor 1991; Whelton et al. 1997) and magnesium (Mervaala et al. 1992; Ascherio et al. 1998) help to prevent the development of hypertension. Epidemiological studies suggest

that milk consumption and dietary intake of dairy proteins are inversely related to the risk of hypertension (McCarron et al. 1984; He and Whelton 1999; Ruidavets et al. 2006). Some intervention studies have shown milk products and dairy proteins to have a blood pressure lowering effect (Appel et al. 1997; Burke et al. 2001). Several milk peptides have been shown to have antihypertensive effects in both animal and clinical studies (for reviews, see FitzGerald et al. 2004; Korhonen and Pihlanto 2006). The most widely studied mechanism underlying the antihypertensive effects of milk peptides is the inhibition of angiotensin-converting enzyme (ACE) (for reviews, see FitzGerald and Meisel 2000; López-Fandiño et al. 2006). The milk-derived peptides have also been shown to have beneficial effects on vascular endothelium and also on arterial tone, but so far, this has been shown only in vitro (Sipola 2002; Sipola et al. 2002b).

The purpose of the present study was to evaluate the long-term blood pressure lowering effects of the *Lactobacillus helveticus* fermented milk (peptide milk) containing the casein-derived tripeptides Isoleucyl-prolyl-proline (Ile-Pro-Pro) and Valyl-prolyl-proline (Val-Pro-Pro) using different blood pressure measurement techniques and to assess the effects of the peptide milk on arterial stiffness in clinical studies. Another purpose was to investigate the antihypertensive mechanisms of Ile-Pro-Pro and Val-Pro-Pro and the kinetics of Ile-Pro-Pro in animal models.

2 REVIEW OF THE LITERATURE

2.1 HYPERTENSION

Hypertension is a common health problem in all Western countries. It is a major risk factor for cardiovascular diseases including coronary heart disease, peripheral artery disease and stroke. Untreated hypertension can therefore cause significant health problems as well as significant costs to society.

2.1.1 Blood pressure and the definition of hypertension

Cardiac output and total peripheral resistance are the factors which finally determine blood pressure. In hypertension, either the cardiac output, the peripheral resistance or both are elevated. Increased fluid volume and increased sympathetic neural system stimulation of the heart raise cardiac output. However, in most hypertensive subjects, the typical haemodynamic finding is normal cardiac output and elevated peripheral resistance (Cowley 1992). The cause of hypertension is unknown in about 95% of cases (essential hypertension). Genetic factors have been estimated to account for about 30% of the variation in blood pressure, and subjects with one or two hypertensive parents have hypertension twice as often as subjects without this genetic background (for review, see Beevers et al. 2001b).

The World Health Organization and the International Society of Hypertension (WHO-ISH 2003) have defined the normal blood pressure values as a systolic blood pressure of less than 130 mmHg and a diastolic blood pressure of less than 85 mmHg. Blood pressure values of 140-159 mmHg (systolic) and 90-99 mmHg (diastolic) are defined as mild hypertension (Whitworth 2003) (*Table 1*). The European Society of Hypertension (ESH) and the European Society of Cardiology (ESC) have the same definition for normal blood pressure as WHO-ISH and

define a systolic blood pressure of 140-159 mmHg and a diastolic blood pressure of 90-99 mmHg as grade 1 hypertension (Mancia et al. 2007). The Joint National Committee (JNC 7), on the other hand, defines normal systolic blood pressure as less than 120 mmHg and diastolic as less than 80 mmHg. In the JNC 7 guidelines, hypertension has been divided into two stages. Stage 1 refers to systolic blood pressure values of 140-159 mmHg or diastolic values of 90-99 mmHg, while stage 2 means systolic blood pressure values of more than 160 mmHg or diastolic values of more than 100 mmHg (Chobanian et al. 2003).

Table 1. Blood pressure classification according to WHO-ISH (Whitworth 2003).

Blood pressure classification	Systolic blood pressure, mmHg	Diastolic blood pressure, mmHg
Optimal	<120	<80
Normal	<130	<85
High-normal	130–139	85–89
Mild hypertension, grade 1	140–159	90–99
Moderate hypertension, grade 2	160–179	100–109
Severe hypertension, grade 3	≥180	≥110

2.1.2 Prevalence of hypertension

The worldwide prevalence of hypertension was approximately 26% in the adult population in 2000 (Kearney et al. 2005). It is estimated that this prevalence is rising among the aging population, along with increasing obesity and less physical activity. The Framingham Heart Study suggests that normotensive subjects aged 55 to 65 have a 90% risk of developing hypertension during their lifetime (Vasan et al. 2002). At the end of 2006, about 500 000 people in Finland used antihypertensive medication, according to the Social Insurance Institution (The Social Insurance Institution 2006). According to the recent National Health and Nutrition Examination Survey (NHANES IV), about 29% of the adult population in the United States are hypertensive (>140/90 mmHg) or use antihypertensive medication (Hajjar and Kotchen 2003). The prevalence of hypertensive subjects in the United States has increased markedly; in the period from 1988 to 1994 (NHANES III), 50 million people were hypertensive, and from 1999 to 2000 (NHANES IV), as many as 65 million (Fields et al. 2004). With 140/90 mmHg as the criterion, 38% of the British adult population are hypertensive (Colhoun et al. 1998).

2.1.3 Hypertension and complications

There is a clear and independent relationship between blood pressure and cardiovascular disease (CVD) events. In recent studies, blood pressure variability has been shown to be a more important determinant of target organ damage than high blood pressure itself (Miao et al. 2006; Tatasciore et al. 2007). In the Framingham Heart Study, the risk ratio for coronary heart disease, stroke, peripheral artery disease and cardiac failure was about 2 to 3 times higher for hypertensive subjects than for normotensive subjects (Kannel 1996; Kannel et al. 2004). Hypertension was also associated with about a four-fold risk of atherothrombotic brain infarction in comparison with normotension (Kannel et al. 1996). Clinical trials have shown that in hypertensive subjects, lowering the blood pressure reduces the risk of cardiovascular diseases (Collins et al. 1990). Decreases in diastolic blood pressure of 5, 7.5 and 10 mmHg have been associated with at least 34%, 46% and 56% reductions, respectively, in the incidence of stroke, and with at least 21%, 29% and 37% lower rates of coronary heart disease (MacMahon et al. 1990). On the other hand, a 20 mmHg increase in systolic blood pressure or a 10 mmHg increase in diastolic blood pressure doubles the risk of CVD in the blood pressure range of 115/75 to 185/115 mmHg (Lewington et al. 2002).

2.1.4 Regulation of blood pressure

In normal conditions, the kidneys play a central role in the long-term regulation of blood pressure, while the central nervous system acts primarily as a short-term regulator (Wyss and Carlson 1999) (*Figure 1*). The central nervous system modulates blood pressure by controlling cardiac output and peripheral resistance. The arterial baroreceptors located at the carotid arch and aortic sinuses and the cardiopulmonary baroreceptors are the two most important neural reflex arches involved in the regulation of blood pressure (Grassi et al. 1998; Stauss 2002). When blood pressure increases, the aortic baroreceptors acutely respond through parasympathetic activation and sympathetic inhibition. Baroreceptor activation decreases as blood pressure falls. When the afferent signals from baroreceptors enter the vasomotor centre in the medulla of the brain, the efferent signals are transferred via sympathetic nerves to the heart, vasculature and kidneys, and the signal is mediated by noradrenaline and adrenaline from the adrenal glands. Both of these catecholamines act by binding to adrenergic receptors (AR) classified as α_1 -AR, α_2 -AR or β -AR. The α_2 -AR family is known to affect vasodilation and vasoconstriction, the response being mediated by one of three receptor subtypes, all of which are present in the brain, kidney, heart and vasculature. Activation of α_{2A} -ARs in the brain lowers blood pressure and plasma noradrenaline levels, while

activation of peripheral α_{2B} -ARs causes sodium retention and vasoconstriction; activation of peripheral α_{2C} -ARs causes cold-induced vasoconstriction (Kanagy 2005).

The kidneys respond to blood pressure changes by altering the excretion of sodium and water and thus controlling extracellular fluid volume (for review, see Lohmeier 2001). In normal conditions, when arterial pressure is elevated, the excretion of sodium and water increases until the blood volume is sufficiently reduced to return the arterial pressure to a normal level. In the long term, normal arterial pressure can be sustained by modulation of the pressure-natriuresis relationship. If the relationship between sodium excretion and arterial pressure is re-established and shifted to higher pressures, hypertension develops (for review, see Navar 1997). The renin-angiotensin-system (RAS) can modify the sensitivity of this pressure-natriuresis system. An increase in sodium intake suppresses the RAS, which enhances the kidneys' ability to excrete sodium and water (for review, see Lohmeier 2001).

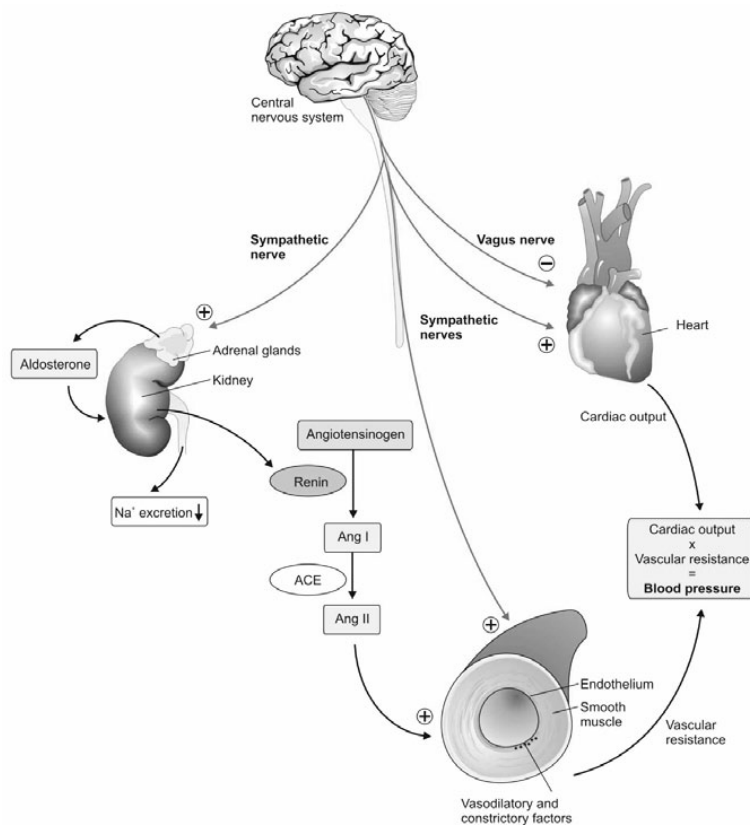


Figure 1. A schematic diagram of the blood pressure regulation system.

2.1.5 Treatment of hypertension

According to the ESH, the ESC, the JNC 7 and the WHO-ISH 2003, the main goal in the treatment of hypertension is to reduce the overall risk of cardiovascular morbidity and mortality or to reduce morbidity and mortality (Chobanian et al. 2003; Whitworth 2003; Mancia et al. 2007).

Lifestyle modification should be included for all patients, especially before antihypertensive medication is prescribed, and also for patients who require pharmacological treatment. Lifestyle modification includes weight reduction in obese and overweight subjects, dietary treatment, physical activity and moderation in alcohol consumption. The optimal body weight has been determined as a body mass index (BMI) of 18.5-24.9, which is the range for normal weight. The recommendation for physical activity is aerobic physical exercise 30 min per day on most days of the week, while the recommended alcohol intake is a maximum of two drinks (e.g. 720 ml of beer or 300 ml of wine) per day for men and one drink for women. Dietary treatment includes sodium restriction and a Dietary Approaches and Stop Hypertension (DASH)-type diet, containing lot of fruit, vegetables and low-fat dairy products (Appel et al. 1997). The DASH diet has been shown to be beneficial for blood pressure as it contains plenty of calcium and potassium (Chobanian et al. 2003).

Pharmacological treatment is needed if the desired blood pressure values cannot be reached with lifestyle modification only. Blood pressure lowering medication reduces the risk of the complications of hypertension. To attain optimal blood pressure levels, some individuals need two or even more antihypertensive drugs. The individual's risk profile and the presence or absence of target organ damage influences the choice of antihypertensive medication. The main classes of antihypertensive medication are *diuretics*, the target organ of which is the nephron, where they interfere with sodium reabsorption; *ACE inhibitors*, which inhibit ACE and therefore reduce the circulating levels of vasoconstrictor angiotensin II; *angiotensin II receptor blockers*, which lower blood pressure by blocking the AT₁ receptors; *adrenergic receptor blockers* (α -blockers, β -blockers): α -blockers cause a modest reduction in both peripheral resistance and cardiac output, β -blockers inhibit β -adrenergic activity and therefore reduce cardiac output and central sympathetic nervous outflow; and *calcium channel blockers*, which reduce intracellular calcium and therefore vasodilate vascular smooth muscle cells (Chobanian et al. 2003). Blood pressure management according to the JNC 7 is presented in *Table 2*.

Table 2. Blood pressure management according to the JNC 7 (Chobanian et al. 2003).

Blood pressure classification	SBP, mmHg		DBP, mmHg	Management
Normal	<120	and	<80	Encourage lifestyle modification
Prehypertension	120–139	or	80–89	Lifestyle modification, no antihypertensive medication if no compelling indication
Stage 1 hypertension	140–159	or	90–99	Lifestyle modification and antihypertensive medication
Stage 2 hypertension	≥160	or	≥100	Lifestyle modification and, for most patients, a combination of two antihypertensive medications

SBP = systolic blood pressure
DBP = diastolic blood pressure

2.1.6 Blood pressure measurement

Blood pressure measurement is challenging, since many different factors regulate blood pressure and the variability of blood pressure in repeated measurements is high. Factors such as measurement variation and biological variation (e.g. ambient, temperature, diurnal, etc.) can affect the accuracy of the measurements. Short-term, daytime, diurnal and seasonal variations all influence blood pressure values. Short-term variability at rest is influenced by respiration and heart rate, and this variability is under the control of the autonomic nervous system. Daytime variability mainly depends on the degree of mental and physical activity. Diurnal variability causes an average fall of 15% in blood pressure during the night. Seasonal variation is considerable, usually with higher blood pressure values during the cold winter than the warm summer (Kristal-Boneh et al. 1996). Some factors can cause an acute increase in blood pressure levels including smoking, caffeine, alcohol, talking, physical activity and psychological factors such as anxiety and white-coat reaction (Reeves 1995). The intake of caffeine, for example, increases the plasma levels of catecholamines, particularly adrenaline (Nurminen et al. 1999). Smoking causes an acute increase in blood pressure and heart rate, which may be mediated via nicotine, which also causes catecholamine release (Primatesta et al. 2001).

Blood pressure can be measured in the office by a physician or nurse, at home by the subjects themselves, or by means of 24-hour ambulatory blood pressure measurement (ABPM). Different blood pressure measurement techniques can produce markedly different blood pressure values, the lowest being in ABPM and the highest in the office blood pressure measurement. This can result in over-treatment of patients with antihypertensive medication (Campbell et al. 2005). In non-pharmacological studies, ABPM and home blood pressure measurements

appear to be useful, because they can detect even small changes in blood pressure values with less observer bias (Kawano 2002).

Office blood pressure measurement is commonly used for the diagnosis of hypertension (Erdine et al. 2006). This measurement has high variability, and all the factors that affect this measurement should therefore be carefully monitored in order to obtain reliable results. Measurements should be made using a validated and calibrated instrument with an appropriate-sized cuff. The patient should not drink coffee, smoke or eat for 30 minutes before the measurement. The patient should rest quietly and refrain from talking for at least five minutes before the measurement. The optimal situation is for the patient to be sitting in a chair with feet on the floor and an arm resting and supported at the heart level (Chobanian et al. 2003). At least two blood pressure measurements should be made during the office visit, and if the difference between the blood pressure values is over 5 mmHg, the measurement should be repeated at least once in order to obtain reliable values.

Home blood pressure measurement is a reliable way to monitor blood pressure values over a long period of time and to follow the effects of treatment. The same criteria for the instrument and the measurement situation applied in office blood pressure measurement settings are also relevant in home blood pressure measurement. Home blood pressure measurements give reliable information about the blood pressure values before antihypertensive medication, and during medication, they give information on the patient's response to treatment (Chobanian et al. 2003).

24-hour ambulatory blood pressure registration (ABPM) is the most reliable method of measuring blood pressure and is regarded as the gold standard. It covers the circadian variation of blood pressure, giving information about the blood pressure values during the daytime and during the sleeping period. Compared with office and home blood pressure measurements, ABPM usually gives the lowest blood pressure values. In most people, blood pressure decreases by about 10 to 20% during the night; failure to do so ("nondippers") indicates an increased risk of cardiovascular diseases (Verdecchia 1995; Pierdomenico et al. 1997). The actual measurements are usually taken every 15 min during the day and every 30 min during the night. This is a valuable method when the patient has suspected white-coat hypertension (Chobanian et al. 2003). The white-coat effect causes normotensive subjects to become hypertensive during a blood pressure measurement. It can be demonstrated using ABPM and sometimes also by using home blood pressure measurement (Beevers et al. 2001a).

2.2 RENIN-ANGIOTENSIN SYSTEM

The RAS is an important regulator of blood pressure and fluid and electrolyte balance (Brown and Vaughan 1998; Schmieder et al. 2007) (*Figure 2*). During the past few years, it has become evident that the RAS, which was originally regarded as a circulating hormone system, is also localised in tissues such as the brain, kidneys, adrenal cortex, heart, blood vessel wall and adipose tissue (for reviews, see Bader et al. 2001; Kramkowski et al. 2006). In the plasma, RAS is determined as a circulating hormone system, which regulates acute changes in the cardiovascular system. In tissues, the RAS regulates long-term changes.

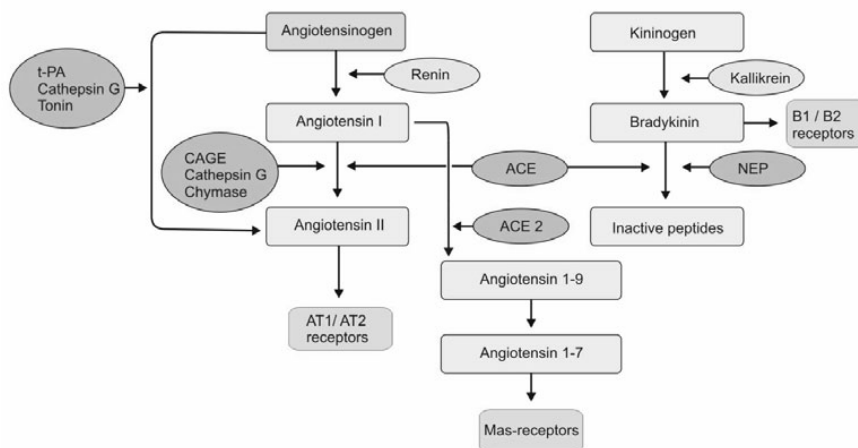


Figure 2. The renin-angiotensin system (Modified from Cheng et al. 2005).

ACE	angiotensin-converting enzyme
ACE2	angiotensin-converting enzyme 2
AT1	angiotensin II type 1 receptor
AT2	angiotensin II type 2 receptor
B1	bradykinin type 1 receptor
B2	bradykinin type 2 receptor
CAGE	chymostatin-sensitive angiotensin II-generating enzyme
NEP	neutral endopeptidase
t-PA	tissue plasminogen activator

The rate-limiting enzyme in the RAS is renin, which is secreted by the juxtaglomerular cells in the kidney. The secretion of renin is regulated by renal perfusion pressure, sodium and sympathetic nerve activity (Hackenthal et al. 1990). In the circulation, renin produces decapeptide angiotensin I (Ang I) from liver-derived angiotensinogen. ACE produces the very potent vasoconstrictor octapeptide angiotensin II (Ang II) from the weak vasoconstrictor Ang I. ACE is also called kininase II, and it catalyses the degradation of the vasodilator bradykinin.

Bradykinin is a nonapeptide formed from kininogens, which are mainly produced by hepatocytes. Bradykinin dilates blood vessels by stimulating the production of nitric oxide (NO), prostacyclin (PGI₂), and endothelium-derived hyperpolarizing factor (EDHF) in the vascular endothelium (Su 2006). It also has direct relaxant effects on vascular smooth muscle via the B₂ receptor (Berguer et al. 1993).

Ang II is a strong direct vasoconstrictor. It induces the release of aldosterone and therefore reduces the excretion of sodium and increases the blood pressure. It increases the activity of the sympathetic nervous system by augmenting noradrenaline release in the vasculature and causing vasoconstriction (for review, see Ardaillou 1997). Ang II also possesses pro-inflammatory properties, which has been emphasised in the development of complications, particularly those associated with hypertension (Mervaala et al. 2000). The effects of Ang II are mediated by the Ang II type 1 (AT₁) and type 2 (AT₂) receptors (for review, see de Gasparo et al. 2000). AT₁ receptors are localised in the vascular smooth muscle cells, brain, heart, kidneys and adrenal cortex. Most of the known cardiovascular effects of Ang II are mediated by AT₁ receptors (Crowley et al. 2007). The activation of AT₁ receptors activates phospholipase C and inhibits adenylate cyclase and thereby increases the levels of intracellular calcium in vascular smooth muscle cells (Orlov et al. 1993; de Gasparo et al. 1995). Ang II causes vasoconstriction also by stimulating the formation of vasoconstrictor endothelin-1 (ET-1) in endothelial cells (Sung et al. 1994; Palatini 2001). AT₂ receptors are expressed at low levels in the adult cardiovascular system, and it has been suggested that AT₂ receptors antagonise the cardiovascular effects of Ang II mediated via AT₁ receptors (for reviews, see Horiuchi et al. 1999; Burnier 2001). The AT₂ receptor-induced vasodilation is mediated via cyclic guanosine monophosphate (cGMP), thus lowering the concentration of intracellular calcium (Savoia et al. 2006).

Ang II mediated hypertension causes end-organ damage via increased blood pressure and also via the direct effects of Ang II. These effects are mediated by AT₁ receptors and lead to end-organ damage in hypertension by promoting inflammation, fibrosis and vascular damage and by stimulating the production of reactive oxygen species. This damage can be reduced by AT₁ receptor blockers and ACE inhibitors (Muller et al. 2000; Luft 2001; Crowley et al. 2006).

There are also other angiotensin peptides, such as the degradation products of Ang II, including Angiotensin III (Ang III), Angiotensin IV (Ang IV) and Angiotensin (1-7) (Ang-1-7) (for review, see Kramkowski et al. 2006). These peptides, too, have biological effects, although Ang II is the major end product of the system. Ang III is formed via the activity of aminopeptidase A, Ang IV via the activity of aminopeptidases A and N, and Ang-(1-7) directly from Ang-(1-9) via the activity of prolylendopeptidase and carboxypeptidases (for review, see Ardaillou

1997). Ang III is a vasoconstrictor that shares most of the clinical properties of Ang II (Ardaillou 1997). It has been reported that Ang IV is a vasodilator and that its effect is mediated by activation of endothelial nitric oxide synthase (eNOS), although the findings are conflicting (Patel et al. 1998). The effects of Ang IV are suggested to be mediated through the AT₄ receptor (Patel et al. 1998). Ang-(1-7) is an active peptide in the RAS and has opposite effects to Ang II. Ang-(1-7) is a vasodilator through stimulation of eNOS activation and NO production via the Mas receptor (Santos et al. 2003; Sampaio et al. 2007) (Figure 2).

In the tissues, Ang II formation (non-ACE) is also catalysed by chymase, cathepsin G, trypsin, chymostatin-sensitive angiotensin II-generating enzyme (CAGE), tissue plasminogen activator (t-PA), and tonin (Kokkonen et al. 1998; Belova 2000; Hu et al. 2003). It has been suggested that relatively high part of human Ang II may be formed in non-ACE pathways in tissues (Hollenberg et al. 1998).

2.3 VASCULAR ENDOTHELIUM

The vascular endothelium – the innermost cell layer covering all vessels – plays a significant role in cardiovascular homeostasis. Endothelium has the ability to sense changes in the mechanical, chemical and humoral environment and to react to these changes with compensatory correction of vascular tone and therefore arterial pressure. The endothelium inhibits platelet adhesion and aggregation, modulates blood flow and regulates vascular tone by releasing vasodilatory substances such as NO, PGI₂ and EDHF as well as vasoconstrictor substances, such as endothelins (ET), thromboxane A₂ (TXA₂) and Ang II. Vasodilator and vasoconstrictor factors have an effect on vascular smooth muscle and thus regulate arterial tone (for reviews, see Vanhoutte and Mombouli 1996; Vapaatalo and Mervaala 2001; Behrendt and Ganz 2002; Vanhoutte et al. 2005) (*Figure 3*).

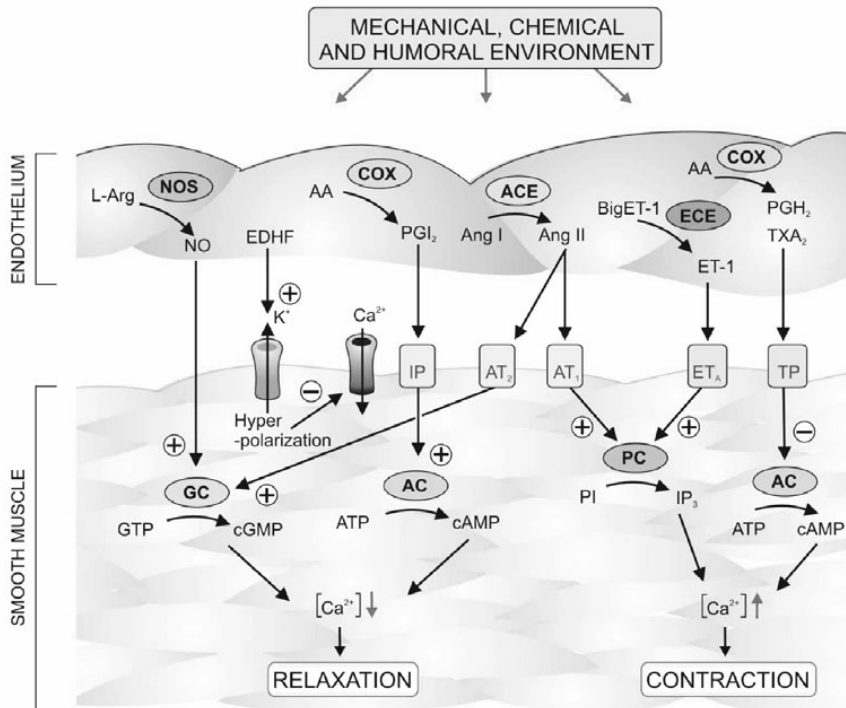


Figure 3. Endothelium-derived vasorelaxing and vasoconstricting factors (Modified from McIntyre et al. 1999; Mombouli and Vanhoutte 1999)

AA	arachidonic acid	ET	endothelin
AC	adenylate cyclase	ET _A	endothelin-1 receptor A
ACE	angiotensin converting enzyme	GC	guanylate cyclase
Ang I	angiotensin I	IP	prostaglandin I ₂ receptor
Ang II	angiotensin II	IP ₃	inositol phosphate
AT ₁	angiotensin type 1 receptor	L-Arg	L-arginine
AT ₂	angiotensin type 2 receptor	NO	nitric oxide
ATP	adenosine triphosphate	NOS	nitric oxide synthase
cAMP	cyclic adenosine- 3', 5'-monophosphate	PC	phospholipase C
cGMP	cyclic guanosine- 3', 5'-monophosphate	PGI ₂	prostacyclin
COX-1	cyclooxygenase 1	PI	phosphoinositol
ECE	endothelin-converting enzyme	TP	thromboxane A ₂ / prostaglandin H ₂ receptor
EDHF	endothelin-derived hyperpolarizing factor	TXA ₂	thromboxane A ₂

2.3.1 Endothelial dysfunction

Endothelial dysfunction has been shown to be related to several cardiovascular diseases and cardiovascular risk factors such as hypertension and dyslipidaemia (for review, see Mombouli and Vanhoutte 1999). Endothelial dysfunction is characterised by a vasoconstrictive and prothrombotic state, meaning a decreased secretion of vasodilatory factors, an increased production and sensitivity to vasoconstrictors, and resistance of vascular smooth muscle to endothelial vasodilators. Dysfunction

is a consequence of an imbalance between relaxing and contracting factors, or growth promoting and inhibiting agents (for review, see Vapaatalo and Mervaala 2001). Inflammation, lipoprotein oxidation or other oxidative stress reactions are factors affecting the development and maintenance of endothelial dysfunction. The endothelium plays a role in inflammatory processes, and the levels of C-reactive protein (CRP) have been shown to increase in endothelial dysfunction (Vita and Loscalzo 2002; Trepels et al. 2006). Theoretically, the clearest and most direct indicators of endothelial dysfunction are NO and its metabolites, as well as PGI₂. In endothelial dysfunction, the NO synthesis is either impaired or inactivated by reactive oxygen species such as superoxide. It has been shown that the balance between NO and superoxide may be even more important than the absolute levels of either of these compounds alone (McIntyre et al. 1999). Thus, because NO is responsible for performing many important actions in the endothelium, such as causing vasodilation and antithrombotic effects, reduced NO formation and activity play a major role in endothelial dysfunction (Busse and Fleming 1996; Raji 2006).

The risk factors for endothelial dysfunction include smoking, hypertension, hyperlipidaemia and diabetes. Reduced NO production is associated with all these risk factors (Bonetti et al. 2003). Aging has also been shown to be related to endothelial dysfunction (Galletta et al. 2006; Taddei et al. 2006). In addition to these traditional risk factors, there are more emerging risk factors, such as physical inactivity, obesity, renal failure and hyperhomocysteinaemia. Endothelial dysfunction can be either a cause or a consequence of several clinical conditions, and it has been suggested that the risk of developing endothelial dysfunction may increase with the number of risk factors present in a subject (Celermajer et al. 1994).

The treatment of endothelial dysfunction includes: reduction of cardiovascular risk factors using antihypertensive medication (e.g. ACE inhibitors, angiotensin II receptor blockers); cholesterol-lowering medication; estrogen replacement therapy in postmenopausal women; folic acid supplementation; reducing smoking and increasing physical activity (Vogel 1999; Hambrecht et al. 2000; Doshi et al. 2001; Vita and Keaney 2001; Taddei et al. 2002). The effect of ACE inhibitors is mainly based on their ability to improve the effect of bradykinin, which enhances the synthesis of nitric oxide in the endothelium. Induced or enhanced nitric oxide production, or added nitrates, can balance insufficient internal NO production (Taddei et al. 2002). ACE inhibitors and AT₁ receptor antagonists may also prevent the age-related impairment of endothelium-dependent hyperpolarisation and relaxation mediated by endothelium-derived hyperpolarizing factor (EDHF) (Kansui et al. 2002).

2.3.2 Endothelium-derived vasodilatory factors

Nitric oxide

Furchgott and Zawadzki were the first to report the presence of a potent vasodilating substance, an endothelium-derived vasodilatory factor that was later shown to be NO (Furchgott and Zawadzki 1980; Ignarro et al. 1987). NO is an active component in the endothelium and is produced from L-arginine in endothelial cells by the enzyme eNOS after stimulation of muscarinic receptors by acetylcholine (Palmer et al. 1988, for review, see Stuehr 1999). Bradykinin also enhances the production of NO. Healthy endothelium constantly releases small amounts of NO.

NO is a very important vasodilator as it relaxes vascular smooth muscle. NO activates soluble guanylate cyclase in smooth muscle cells to produce cGMP, which causes smooth muscle relaxation by reducing intracellular calcium content (Feletou and Vanhoutte 2000; Behrendt and Ganz 2002). In addition to vasodilation, NO also regulates many physiological activities, including antithrombotic actions and immune cell activity (Gewaltig and Kojda 2002). These effects play a significant role in protecting against vascular injury (for review, see Hobbs et al. 1999). Inflammation impairs the expression and bioavailability of eNOS in experimental animals and in humans (for review, see Huang and Vita 2006). Oxidative stress decreases eNOS expression and the bioavailability of NO (Liao et al. 1995).

Prostacyclin

PGI₂ is a prostanoid which causes vasodilation and inhibits platelet aggregation. PGI₂ is synthesised from arachidonic acid by cyclooxygenases 1 and 2 (COX-1 and COX-2). Most tissues express COX-1 constitutively, but COX-2 expression rises markedly mainly with cell activation, as in inflammation (Maclouf et al. 1998). Endothelial COX-2 produces mainly PGI₂ in humans (FitzGerald and Patrono 2001; Rudic et al. 2005). Stimulation of prostaglandin I₂ (IP) receptors activates adenylate cyclase in smooth muscle to produce cyclic adenosine monophosphate (cAMP) from adenosine triphosphate. cAMP decreases the concentration of intracellular calcium and causes vasodilation (Cohen and Vanhoutte 1995; FitzGerald and Patrono 2001). The synthesis of PGI₂ in endothelial cells is activated by factors such as shear stress and hypoxia (for review, see Lüscher and Noll 1995).

Endothelium-derived hyperpolarizing factor

Endothelium-dependent relaxation, which is resistant to the inhibition of nitric oxide synthase (NOS) and COX, is thought to be mediated via EDHF (Cohen and Vanhoutte 1995). EDHF mediates vasodilation by opening the Ca²⁺-regulated K⁺

channels in the vascular smooth muscle cells and by inducing hyperpolarisation in smooth muscle membrane and thus inhibiting the inflow of calcium. Because of this, the concentration of intracellular calcium decreases, which causes relaxation in the smooth muscle (for reviews, see Feletou and Vanhoutte 1999; Feletou and Vanhoutte 2006). EDHF-mediated vasodilation plays an important role when NO production is impaired (Bauersachs et al. 1996). The chemical identity of EDHF has received a lot of attention, but no consensus has been reached. Several major candidates for EDHF have been proposed, such as K^+ , and arachidonic acid metabolites, such as epoxy-eicosatrienoic acid (for review, see Feletou and Vanhoutte 2006). One very recent candidate is C-type natriuretic peptide (Sandow and Tare 2007).

2.3.3 Endothelium-derived vasoconstrictory factors

Angiotensin II

Angiotensin II has been described earlier in section 2.2.

Endothelin-1

ETs, including ET-1, ET-2 and ET-3, are peptides with vasoconstrictor properties. ET-1, which comprises 21 amino acids, is a potent vasoconstrictor and its production is catalysed by endothelin-converting enzyme (ECE). Low shear stress, hypoxia, stretching the vascular wall and substances such as noradrenaline and Ang II increase the production of ET-1 (for review, see Lüscher and Noll 1995). The vascular effects of ET-1 are mediated via the ET_A receptor present in smooth-muscle cells, and via the ET_B receptor present mainly in the endothelium (for reviews, see Rothermund and Paul 1998; Neylon 1999). The stimulation of ET_A receptors activates phospholipase C and thereby increases the formation of inositol triphosphate, which increases the levels of intracellular calcium and causes vasoconstriction (Taddei et al. 2000) (*Figure 3*).

Prostanoids

In addition to PGI_2 , the endothelium also produces vasoconstrictor prostanoids, such as prostaglandin H_2 (PGH_2), TXA_2 and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). These vasoconstrictors are also produced by COX. Prostaglandin H_2 is further converted to PGI_2 by PGI_2 synthase (Cohen 1995).

2.4 MINERALS AND BLOOD PRESSURE

Sodium is known to have harmful, blood pressure increasing effects, whereas calcium, potassium and magnesium are beneficial for blood pressure and have an important nutritional role in controlling blood pressure (for reviews, see Massey 2001; Karppanen et al. 2005).

2.4.1 Sodium

The harmful effects of sodium on blood pressure are well known. It has been shown in epidemiological and clinical trials that high sodium intake increases blood pressure (for review, see Karppanen and Mervaala 2006). In the Intersalt study – the largest epidemiological study of its kind – elevated urinary sodium excretion as a marker of high sodium intake was associated with higher systolic and diastolic blood pressures (Intersalt Cooperative Research Group 1988; Elliott et al. 1996). Several meta-analyses have shown that restricting sodium intake reduces blood pressure (Cutler et al. 1997; Graudal et al. 1998; He and MacGregor 2002; Geleijnse et al. 2003). In a meta-analysis of 17 trials with hypertensive subjects, a daily reduction of 6 g of salt reduced systolic blood pressure by about 7 mmHg and diastolic blood pressure by about 4 mmHg (He and MacGregor 2002). In a meta-analysis of 40 trials, an average daily sodium reduction of 1.8 g reduced systolic blood pressure in hypertensive subjects by an average of 5.2 mmHg and diastolic blood pressure by 3.7 mmHg. In normotensive subjects, the mean reduction in systolic blood pressure was 1.3 mmHg and in diastolic blood pressure 1.1 mmHg (Geleijnse et al. 2003).

In the second DASH trial, a reduction in sodium intake together with a diet rich in potassium, magnesium and calcium reduced systolic blood pressure compared to the control diet by 7.1 mmHg in normotensive subjects and by 11.5 mmHg in hypertensive subjects (Sacks et al. 2001).

2.4.2 Potassium

Experimental, epidemiological and clinical trials show that potassium may have beneficial effects on blood pressure (for reviews, see Kotchen and McCarron 1998; He and MacGregor 2001; He and MacGregor 2003). In a meta-analysis of 19 clinical trials (586 participants), oral potassium supplement (average 3.4 g daily) lowered systolic (-5.9 mmHg) and diastolic blood pressures (-3.4 mmHg) (Cappuccio and MacGregor 1991). In another meta-analysis of 33 studies (2609 participants), an increased intake of potassium reduced systolic blood pressure by an average of 3.1 mmHg and diastolic blood pressure by 2 mmHg (Whelton et al. 1997). The blood pressure lowering effect of potassium has been shown to be more

marked in hypertensive subjects. In a meta-analysis of 27 trials, a mean potassium intake of 1700 mg/d reduced systolic blood pressure in hypertensive subjects by an average 3.5 mmHg and diastolic blood pressure by 2.5 mmHg. In normotensive subjects, the mean reduction in systolic blood pressure was 1.0 mmHg and that in diastolic blood pressure 0.3 mmHg (Geleijnse et al. 2003).

The antihypertensive effect of potassium seems to be multifactorial (for reviews, see Treasure and Ploth 1983; Barri and Wingo 1997). The blood pressure lowering effect of potassium may be related to its diuretic properties. Potassium causes natriuresis and therefore lowers the extracellular fluid volume and reduces blood pressure (Young et al. 1976; Mervaala et al. 1992). Alternatively, potassium may also reduce the activity of the sympathetic nervous system, decrease renin production and the response to noradrenaline and Ang II (Campbell and Schmitz 1978; Fujita and Sato 1992; Vaskonen 2003). It has also been shown that potassium has effects on vascular responses, and it improves endothelium-dependent and -independent relaxation (Mervaala et al. 1994; for review, see Mervaala et al. 1998a).

2.4.3 Calcium

The antihypertensive activity of calcium has been clearly demonstrated in experimental studies (for review, see Hatton and McCarron 1994). In studies on SHR, calcium supplements have prevented the elevation of blood pressure (Pörsti et al. 1990; Pörsti et al. 1991; Mäkynen et al. 1995; Schleiffer and Gairard 1995). On the other hand, it has been observed that the removal of dietary calcium leads to a rise in blood pressure in experimental animals (Schleiffer and Gairard 1995).

McCarron et al. were the first to report a connection between low intake of calcium and elevated blood pressure in humans (NHANES I) (McCarron et al. 1984). Several epidemiological studies demonstrating a connection between calcium intake and blood pressure have since been published (Cappuccio et al. 1995; Narayan et al. 1998; Ruidavets et al. 2006; for review, see Geleijnse and Grobbee 2000). Beneficial effects of calcium on blood pressure have been also demonstrated in several clinical trials (Allender et al. 1996; Bucher et al. 1996; Griffith et al. 1999; van Mierlo et al. 2006). According to a recent meta-analysis of 40 studies, calcium supplementation (mean 1200 mg/d) reduced systolic blood pressure by about 2 mmHg and diastolic blood pressure by about 1 mmHg (van Mierlo et al. 2006).

A diet rich in calcium, potassium, magnesium and fibre and poor in saturated fatty acids (e.g. diet rich in fruits, vegetables and low-fat dairy products) has been shown to reduce blood pressure in the DASH trial (Appel et al. 1997). With the DASH diet, the mean reduction in systolic blood pressure was -5.5 mmHg and in diastolic blood pressure -3.0 mmHg more than in the control group. In persons

with elevated blood pressure (140 mmHg/90 mmHg), the DASH diet produced a considerably higher reduction in blood pressure. Compared with the control group, the mean reduction in systolic blood pressure was about 11 mmHg greater and the mean reduction in diastolic blood pressure about 6 mmHg greater (Appel et al. 1997).

Calcium may influence blood pressure by several mechanisms. One of the antihypertensive mechanisms is its natriuretic effect, which increases the excretion of salt (for reviews, see Hatton and McCarron 1994; Zemel 2001). Calcium plays an important role in controlling the tension of vascular smooth muscle. Extracellular calcium increases the stability of the cell membrane in vascular smooth muscle and reduces ion penetration through the cell membrane, which causes a decrease in intracellular calcium and vasodilation (Hatton et al. 1995). Potassium outflow is also reduced by a calcium-rich diet. Reduced cellular outflow of potassium is typical of calcium-induced cell membrane stabilisation (Furspan et al. 1989). The concentration of extracellular calcium may influence blood pressure by increasing the formation and release of endothelium-derived relaxing factors, such as NO and PGI₂. Calcium has also been shown to reduce blood pressure by downregulating renal ACE (Pörsti et al. 2004).

The effects of calcium on blood pressure may partly be mediated by calcium-controlling hormones, including parathyroid hormone (PTH), 1.25(OH)₂ vitamin D, and possibly calcitonin. Many hypertensive patients have elevated PTH levels, and increased dietary calcium and a concurrent reduction of sodium intake may therefore reduce blood pressure by reducing PTH levels. PTH influences blood pressure by increasing the formation and release of 1.25(OH)₂ vitamin D, which independently increases blood pressure by increasing the amount of intracellular calcium in vascular smooth muscle. An increased concentration of intracellular calcium increases cellular contractility and raises blood pressure values (Zemel 2001; Vaskonen 2003). Dietary calcium reduces the production of 1.25(OH)₂ vitamin D and therefore also the cellular intake of calcium. Calcium is also believed to influence the activity of the sympathetic nervous system. In experimental studies, the deprivation of dietary calcium led to an increase in noradrenaline and adrenaline levels, while calcium supplements were found to reduce the concentration of adrenaline in SHR (Hagihara et al. 1990; Scrogin et al. 1991).

2.4.4 Magnesium

Like potassium and calcium, magnesium has also beneficial effects on blood pressure, even though the results are somewhat controversial (for review, see Sontia and Touyz 2007).

Experimental trials have shown that magnesium administration returned systolic blood pressure to normal or attenuated the development of hypertension (Mervaala et al. 1997; Soltani et al. 2007). Magnesium and potassium intake have been shown to reduce the harmful effects of sodium and to reduce blood pressure in SHR (Mervaala et al. 1992). On the other hand, magnesium deficiency has been shown to increase blood pressure in rats (Blache et al. 2006).

In a meta-analysis of 20 studies magnesium reduced systolic blood pressure by an average of -0.6 mmHg and diastolic blood pressure by -0.8 mmHg (Jee et al. 2002). When the magnesium dose was increased, a dose-dependent reduction was seen in blood pressure values. In a ten-year follow-up study, magnesium intake was inversely associated with the risk of developing hypertension (Song et al. 2006). Some clinical studies support the claim that magnesium deficiency plays a role in the development of hypertension (Touyz et al. 1992; Resnick et al. 1997).

It has been suggested that magnesium has beneficial effects on blood pressure via its vasodilatory properties. Magnesium acts as a calcium channel antagonist and stimulates the production of vasodilatory PGI₂ and NO (for review, see Sontia and Touyz 2007).

2.5 BIOACTIVE PEPTIDES DERIVED FROM MILK PROTEINS

Milk proteins are an important source of bioactive peptides. Biologically active peptide fragments are formed when the milk proteins, whey and casein, are broken down by digestive enzymes in the gastrointestinal tract, by fermentation of milk with proteolytic starter culture or in proteolysis by enzymes derived from micro-organisms (Teschemacher et al. 1997; Meisel 1998; Korhonen and Pihlanto 2003a; Korhonen and Pihlanto 2003b). Lactic acid bacteria and their proteolytic activity are well characterised. Lactic acid bacteria e.g. *Lactobacillus helveticus*, *Lactococcus lactis* and *Lactobacillus delbrueckii* ssp. *bulgaricus* are traditionally used in the production of fermented dairy products (Nakamura et al. 1995a; Nakamura et al. 1995b; Christensen et al. 1999). Over the last decade, many strains of lactic acid bacteria have been demonstrated to release bioactive peptides from milk proteins during milk fermentation (Nakamura et al. 1995a; Nakamura et al. 1995b; Christensen et al. 1999; FitzGerald and Murray 2006; Korhonen and Pihlanto 2006). Bioactive peptides have been shown to have many functions. These include their effects on the gastrointestinal system, including the regulation of digestive enzymes and the subsequent modulation of nutrient absorption from the intestine (Shimizu 2004). Peptides may also enhance immune cell functions and thereby affect the immune

system, while peptides with opioid activity affect the nervous system (for reviews, see Gill et al. 2000; Teschemacher 2003; Korhonen and Pihlanto 2006). Peptides also influence the cardiovascular system. The most studied blood pressure lowering mechanism of milk peptides is ACE inhibition (Nakamura et al. 1995a; Nakamura et al. 1995b; Takano T 1998; Sipola et al. 2002a; for review, see Jauhiainen and Korpela 2007). Milk peptides may also lower blood pressure via other mechanisms, such as via opioid-like activities (Meisel and FitzGerald 2000). When the milk peptides are taken as a part of milk products, milk minerals might also have a role in lowering blood pressure.

2.5.1 Peptides and blood pressure

■ Animal studies

Acute effects

Most animal studies on milk-derived peptides are acute (short-term) experiments, in which the effects on blood pressure are evaluated after a single oral administration. The milk casein-derived tripeptides Ile-Pro-Pro and Val-Pro-Pro (*Figure 4*) have been shown to reduce blood pressure in SHR after a single oral administration (Nakamura et al. 1995b). A milk product fermented by *Lactobacillus helveticus* bacteria and *Saccharomyces cerevisiae* yeast containing Ile-Pro-Pro and Val-Pro-Pro peptides reduced systolic blood pressure within six to eight hours of oral administration in SHR. The antihypertensive effect in this study was seen only with the hypertensive rat model, and not in normotensive Wistar Kyoto rats (Nakamura et al. 1995b). It has been shown that whey protein-based α -lactorphin (Tyr-Gly-Leu-Phe) lowers blood pressure dose-dependently after oral feeding in SHR and in normotensive Wistar Kyoto rats. Blood pressure was measured by continuous radiotelemetric monitoring, and the decrease in blood pressure reached its maximum 50-100 minutes after administration and returned to the baseline level after 200 minutes (Nurminen et al. 2000). Milk fermented with two *Lactobacillus helveticus* strains, *Lactobacillus helveticus* CHCC637 and *Lactobacillus helveticus* CHCC641 reduced mean arterial pressure by about 12 mmHg more than the control product in SHR (Fuglsang et al. 2002). Tyr-Pro dipeptide from a *Lactobacillus helveticus* CPN4 fermented yoghurt-like product also showed acute antihypertensive effects in SHR after a single oral dose when systolic blood pressure was measured by the tail-cuff method (Yamamoto et al. 1999).

Long-term effects

Ile-Pro-Pro and Val-Pro-Pro tripeptides have been shown to attenuate the development of hypertension in SHR after long-term, 12- and 13-week oral feeding (Sipola et al. 2001; Sipola et al. 2002a). At the end of the 12-week treatment period, systolic blood pressure was 17 mmHg lower in the group that received *Lactobacillus helveticus* LBK-16H fermented milk containing Ile-Pro-Pro and Val-Pro-Pro as compared to the control group, which was given water, and 12 mmHg lower in the group that was given the same tripeptides in water (Sipola et al. 2001). Similar results were obtained in the 13-week study; fermented milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptides attenuated the development of hypertension (Sipola et al. 2002a). The results of the animal studies are summarised in *Table 3*.

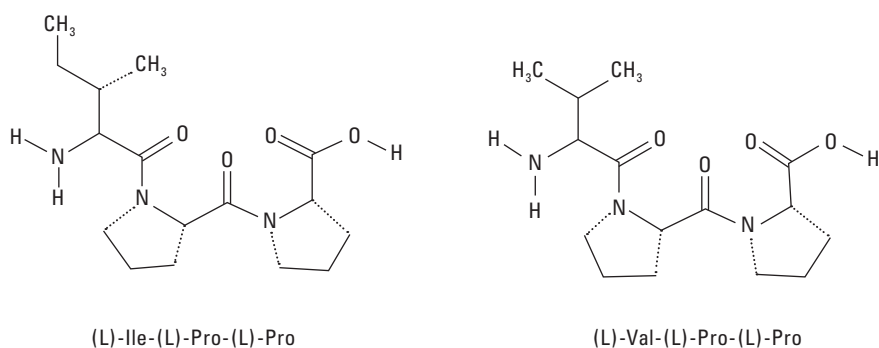


Figure 4. Structural formulas of Ile-Pro-Pro and Val-Pro-Pro.

Table 3. Animal studies on the effect of milk-derived bioactive peptides on SBP in SHR.

Duration	Product	Peptide dose mg/kg of body weight	Reduction in SBP	Reference
Acute experiments				
10 h	Casein hydrolysates	15	Maximal reduction -22 mmHg after 6 h.	Yamamoto et al. 1994
24 h	Fermented milk containing tripeptides IPP and VPP and pure peptides	IPP 0.3 and VPP 0.6	Fermented milk: maximal reduction -22 mmHg after 6 h. IPP: maximal reduction -28 mmHg after 8 h. VPP: -32 mmHg after 4 h.	Nakamura et al. 1995b
24 h	Lys-Val-Leu-Pro-Val-Pro-Gln from β -casein	2	Maximal reduction -32 mmHg after 6 h.	Maeno et al. 1996
24 h	Ile-Pro-Ala from whey protein	8	-31 mmHg.	Abubakar et al. 1998
24 h	Tyr-Pro from yoghurt-like product	1	Dose-dependent effect, maximal reduction -27 mmHg after 6 h.	Yamamoto et al. 1999
200 min	α -Lactorphin	0.1	Dose-dependent effect. -23 mmHg vs. control. Maximal reduction after 50–100 min.	Nurminen et al. 2000
8 h	Fermented milk containing ACE inhibitors	Not reported	-11–(-12) mmHg (mean arterial pressure) vs. control.	Fuglsang et al. 2002
24 h	Ala-Leu-Pro-Met	Not reported	Maximal reduction 21 mmHg after 8 h.	Murakami et al. 2004
Long-term experiments				
4 wk	Tryptic hydrolysate of milk casein	2040	-14 mmHg compared to control.	Karaki et al. 1990
12 wk	Fermented milk containing tripeptides IPP and VPP and pure peptides	Fermented milk: IPP 3.5 and VPP 3.5. Peptide: IPP 2.2 and VPP 2.2.	Fermented milk: -17 mmHg and pure tripeptides -12 mmHg vs. control	Sipola et al. 2001
14 wk	Fermented milk containing tripeptides IPP and VPP	IPP 1.3 and VPP 2.0	-21 mmHg vs. control.	Sipola et al. 2002a

IPP = Ile-Pro-Pro

SBP = systolic blood pressure

SHR = spontaneously hypertensive rats

VPP = Val-Pro-Pro

■ Clinical studies

Lactobacillus helveticus fermented milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptides has been shown to lower systolic and diastolic blood pressure in hypertensive subjects (Hata et al. 1996; Seppo et al. 2002; Mizushima et al. 2004). In two Japanese placebo-controlled studies on hypertensive subjects, *Lactobacillus helveticus* and *Saccharomyces cerevisiae* fermented milk reduced systolic and diastolic blood pressure during eight-week (Hata et al. 1996) and four-week (Mizushima et al. 2004) interventions. In an eight-week placebo-controlled study on 17 hypertensive subjects, systolic and diastolic blood pressures decreased more (-10 mmHg and -7 mmHg respectively) in the group receiving *Lactobacillus helveticus* fermented milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptides than in the placebo group receiving normal fermented milk (Seppo et al. 2002).

Tablets containing Ile-Pro-Pro and Val-Pro-Pro tripeptides have also been shown to have a tendency to lower blood pressure in hypertensive subjects (Aihara et al. 2005; Mizuno et al. 2005). When comparing the studies with fermented milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptides and the studies with tablets containing these tripeptides, it can be concluded that the p value between the groups has been reported in about half of the studies, and reported compared to the baseline level in the other half. The absolute blood pressure reductions with fermented milk and with tablets are about the same.

In a very recent clinical trial with casein hydrolysate, systolic and diastolic blood pressure decreased more in the casein group than in the placebo group (Cadee et al. 2007). In a recent study of milk with whey peptides, the test product did not reduce systolic or diastolic blood pressure (Lee et al. 2007). Milk fermented by *Lactobacillus casei* and *Lactococcus lactis* and containing γ -aminobutyric acid reduced blood pressure during a 12-week treatment period. Systolic blood pressure decreased about 17 mmHg from baseline, which was significantly more than the reduction in the placebo group. Diastolic blood pressure in the fermented milk group did not differ from the placebo group (Inoue et al. 2003). In another eight-week study, systolic blood pressure was significantly lower in the group receiving yoghurt fermented with two strains of *Streptococcus thermophilus* and two strains of *Lactobacillus acidophilus* as compared to the group which received yoghurt fermented with two strains of *Streptococcus thermophilus* and one strain of *Lactobacillus rhamnosus*. A similar lowering of systolic blood pressure was also obtained in a group which received yoghurt fermented with two strains of *Streptococcus thermophilus* and one strain of *Enterococcus faecium* (Agerholm-Larsen et al. 2000). Almost all the clinical trials with bioactive milk peptides involve active and placebo groups. The results of clinical studies are presented in Table 4.

2.5.2 Blood pressure lowering mechanisms

Angiotensin-converting enzyme inhibition

The most widely studied mechanism by which milk-derived peptides can reduce blood pressure is ACE inhibition (for reviews, see FitzGerald and Meisel 2000; Kitts and Weiler 2003; López-Fandiño et al. 2006).

Several antihypertensive peptides that inhibit ACE have been identified from milk products, and the ACE inhibitory activity and IC_{50} values (the concentrations at which ACE activity *in vitro* is inhibited by 50%) of some of these peptides have been determined *in vitro* (Karaki et al. 1990; Nakamura et al. 1995b; Maeno et al. 1996; Yamamoto et al. 1999). The IC_{50} values of the casein-derived tripeptides Ile-Pro-Pro and Val-Pro-Pro have been found to be 5 μ M and 9 μ M, respectively. The ACE inhibitory activity of these peptides is, however, much weaker than that of ACE inhibitor medication (Mullally et al. 1996), and it cannot always be directly related to the blood pressure lowering effects *in vivo*. The relationship between ACE inhibitory peptides and the structural activity has not been confirmed. However, some general features have been found. ACE inhibitory peptides usually contain 2-12 amino acids (Robert et al. 2004), and hydrophobic amino acids at the C-terminal position, as in proline, could be the most likely to have ACE inhibitory properties (Ondetti and Cushman 1984).

The ACE inhibitory activity of Ile-Pro-Pro and Val-Pro-Pro has been shown *in vitro* (Nakamura et al. 1995a), and evidence for ACE inhibition has also been demonstrated *in vivo*. *Lactobacillus helveticus* fermented milk containing Ile-Pro-Pro and Val-Pro-Pro raised plasma renin activity in SHR during long-term treatment (Sipola et al. 2002a). ACE activity in the aorta of SHR was reduced after a single oral administration and after long-term treatment with *Lactobacillus helveticus* and *Saccharomyces cerevisiae* fermented milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptides (Nakamura et al. 1995b; Nakamura et al. 1996). ACE activity has also been studied in a clinical trial, but no clear ACE inhibition was found. In a study by Mizushima et al., the serum levels of Ang I and Ang II were measured but there was no significant change in the Ang I/Ang II ratio at four weeks (Mizushima et al. 2004). Milk-derived peptides such as α -lactorphin and β -lactorphin, which have antihypertensive effects via other mechanisms than ACE inhibition, have also been investigated (Nurminen et al. 2000).

In addition to local ACE, Ang II formation can also be related to other enzymes like CAGE, cathepsin G, chymase, neutral endopeptidase (NEP), t-PA and tonin (Figure 2.). These enzymes may also play a role when the blood pressure lowering effects of biologically active peptides are considered.

Table 4. Clinical studies on the effect of milk derived bioactive peptides on SBP and DBP.

Product	Subjects (n)	Duration (weeks)	Peptide dose, mg/d	Results	Reference
Fermented milk containing tripeptides IPP and VPP	30	8	IPP 1.1 and VPP 1.5	SBP -14 mmHg ($p<0.01$) and DBP -7 mmHg ($p<0.05$). Differences between the groups not reported.	Hata et al. 1996
Tryptic hydrolysate of casein	18	4	200	DBP -7 mmHg. No placebo group.	Sugai 1998
Fermented milk containing whey protein	20	8	Whey protein 8.8	SBP decreased vs. baseline level ($p<0.05$). Differences between the groups not reported.	Kawase et al. 2000
Fermented yoghurt	70	8	Not reported.	SBP ($p<0.05$) decreased vs. placebo.	Agerholm-Larsen et al. 2000
Fermented milk containing tripeptides IPP and VPP	18 + 26 ^a	8	Not reported.	SBP -12 mmHg. No effects in normotensive subjects.	Itakura et al. 2001 *
Tablets containing tripeptides IPP and VPP	81	8	IPP 1.64 and VPP 2.52	SBP -12 mmHg and DBP -8 mmHg. Differences between the groups not reported.	Kajimoto et al. 2001a *
Fermented milk containing tripeptides IPP and VPP	30	8	IPP 1.52 and VPP 2.53	SBP -14 mmHg and DBP -7 mmHg, ($p<0.05$) vs placebo.	Kajimoto et al. 2001b *
Tablets containing tripeptides IPP and VPP	20	1	IPP 11.5 and VPP 17.7	SBP -3 mmHg and DBP -1 mmHg.	Yasuda et al. 2001 *
Fermented milk containing tripeptides IPP and VPP	17	8	IPP 2.25 and VPP 3-3.75	SBP -7.3% ($p=0.05$) and DBP -7.3% ($p<0.05$) vs placebo.	Seppo et al. 2002
Whey protein isolate hydrolysate	30	6	20000 (20g)	SBP-11 mmHg and DBP -7 mmHg vs. placebo.	Pins and Keenan 2003
Fermented milk containing peptides SKVVP	29	4	Not reported.	SBP -9 mmHg. Differences between the groups not reported.	Ashar and Chand 2004

Fermented milk containing tripeptides IPP and VPP	46	4	IPP 1.2 and VPP 2.0	SBP -5 mmHg ($p=0.039$) and DBP -2 mmHg ($p>0.05$).	Mizushima et al. 2004
Casein hydrolysate (C12 peptide)	10	single dose	maximal C12 peptide 200	SBP -5mmHg and DBP -7 mmHg.	Townsend et al. 2004
Fermented milk containing tripeptides IPP and VPP	60/39 ^b	8–10-wk period 1+ 5–7-wk period 2	IPP 2.4–2.7 and VPP 2.4–2.7	SBP -6.4 mmHg ($p=0.10$) and DBP -1.5 mmHg ($p=0.41$) vs. placebo (at the end of period 2)	Tuomilehto et al. 2004
Tablets containing powdered fermented milk, IPP and VPP	40 + 40 ^c	4	IPP 4.7 and VPP 8.3	In high normal blood pressure group, SBP -3 mmHg ($p=0.27$) and DBP -5 mmHg ($p=0.045$) vs. placebo. In mild hypertension group, SPB -11 mmHg ($p=0.003$) and DBP -7 mmHg ($p=0.055$) vs. placebo.	Aihara et al. 2005
Tablets containing IPP and VPP	131	6	IPP+VPP 1.8–3.6	SBP decreased dose-dependently (maximal -13 mmHg) and vs. placebo ($p=0.001$). DBP decreased dose-dependently.	Mizuno et al. 2005
Whey protein isolate hydrolysate	30	6	20000 (20g)	SBP -8.0 mmHg ($p<0.05$) and DBP -5.5 mmHg ($p<0.05$) vs. placebo	Pins and Keenan 2006
Casein hydrolysate (C12 peptide)	48	4	C12 peptide 3800	SBP -11 mmHg and DBP -7 mm Hg. Differences between the groups not reported.	Cadee et al. 2007
Milk with whey peptides.	54	12	3250	No differences in blood pressure values in peptide group.	Lee et al. 2007

*Translated into English.

^a18 hypertensive and 26 normotensive subjects.

^b60 subjects participated in period 1 and 39 subjects in period 2.

^c40 subjects with high normal blood pressure and 40 with mild hypertension.

DBP = diastolic blood pressure

IPP = Ile-Pro-Pro

SBP = systolic blood pressure

VPP = Val-Pro-Pro

Other possible mechanisms

Several milk peptides have opioid-like activity (for reviews, see Antila et al. 1991; Teschemacher and Koch 1991; Teschemacher et al. 1997). Opioid-like activity has been discovered in many peptide fragments from casein, and the first opioid milk peptide agonist to be characterised was derived from β -casein (β -casomorphin). The peptides with opioid-like activity derived from α -casein are called α -exorphins, while those from κ -casein are called casoxins. Opioid peptides can also be derived from the whey proteins α -lactalbumin and β -lactoglobulins. α -Lactorphin, found in α -lactalbumin, has been shown to lower blood pressure in SHR. Because the antihypertensive effect of α -lactorphin was completely prevented by the opioid receptor antagonist naloxone, it has been proposed that the antihypertensive effect may be mediated via opioid receptors (Nurminen et al. 2000).

Milk-derived caseinophosphopeptides have been shown to increase the solubility of calcium and to enhance its absorption (Tsuchita et al. 2001). However, recent clinical studies suggest that the enhancement of calcium absorption by caseinophosphopeptides is so minor that is unlikely to have any clinical relevance (Lopez-Huertas et al. 2006; Teucher et al. 2006). Bioactive peptides may also have beneficial cardiovascular effects via their antithrombotic effects. The peptides that inhibit the aggregation of blood platelets have been identified as caseinomacropепptides (for review, see Fiat et al. 1993).

2.5.3 Peptides and vascular function

Milk-derived peptides might have beneficial effects on blood pressure by improving vascular function. However, the effect has been shown only in *in vitro* studies. α -lactorphin and β -lactorphin derived from whey protein have been shown to improve arterial function in SHR *in vitro* (Sipola et al. 2002b). Fermented milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptides has also been shown to improve sodium nitroprusside-induced endothelium-independent vasorelaxation in SHR *in vitro* (Sipola 2002). This effect of Ile-Pro-Pro and Val-Pro-Pro might be related to these peptides' weak ACE-inhibitory activity, because in many studies, ACE inhibitors have been shown to reduce arterial stiffness in rats and in humans (Chillon et al. 1992; Ahimastos et al. 2005). Casomokinin L has also been shown to relax mesenteric arteries in an experimental trial (Fujita et al. 1996), and the dipeptide Val-Tyr has been reported to cause vascular relaxation in the aorta *in vitro* in SHR (Tanaka et al. 2006). Bovine lactoferrin, on the other hand, has been shown to cause hypotension in rats via an endothelium-dependent vasodilation that is strongly mediated by NO production (Hayashida et al. 2004).

2.5.4 Kinetics of peptides

The kinetics of milk-derived bioactive peptides are not well known. However, the kinetics of peptides are a central issue in the evaluation of their effects and efficacy. Milk peptides which are not degraded in proteolysis can theoretically be absorbed intact. It has been suggested that dipeptides and tripeptides are absorbed in the intestine (Masuda et al. 1996; Satake et al. 2002). Furthermore, it has also been reported that tripeptides containing a C-terminal proline-proline bond are usually resistant to human proteolytic enzymes (for review, see Vanhoof et al. 1995). Val-Pro-Pro has been reported to be transported via the Caco-2 cell monolayer by paracellular diffusion (Satake et al. 2002). In a recent study, the absorption of Ile-Pro-Pro was evaluated by measuring the plasma concentrations of peptides in humans before and after the intake of tripeptides. The plasma concentrations of Ile-Pro-Pro increased, and the net area under the curve (AUC) for plasma Ile-Pro-Pro during the 120-minute follow-up after consumption of lactotripeptide enriched yoghurt (20.4 mg Ile-Pro-Pro, 20 mg Val-Pro-Pro, 16.5 mg Leu-Pro-Pro) in the fasted state was 2.2-fold compared to the placebo values. It was therefore suggested that Ile-Pro-Pro was absorbed through the intestine and reached the circulation undegraded (Foltz et al. 2007). The absorption of longer peptides has also been studied. The ACE-inhibitory peptide lactokinin (Ala-Leu-Pro-Met-His-Ile-Arg) has been found to be transported intact through the Caco-2 Bbe monolayer *in vitro*. Ala-Leu-Pro-Met-His-Ile-Arg was clearly shown to be present in mucosal compartment when analysed with high performance liquid chromatography and mass spectrometry, but in serosal compartment it was detected only with mass spectrometry (Vermeirssen et al. 2002).

3 AIMS OF THE STUDY

Nutritional factors have been shown to have important role in the prevention and treatment of hypertension. Earlier studies have shown that minerals, such as sodium, potassium, calcium and magnesium have a role in blood pressure regulation. Recent experimental and clinical studies have suggested that the bioactive peptides Ile-Pro-Pro and Val-Pro-Pro derived from milk have beneficial effects on blood pressure. However, the antihypertensive mechanisms, the kinetics, the long-term blood pressure lowering effects and the effects of vascular function of Ile-Pro-Pro and Val-Pro-Pro are not well known. The present study investigated these properties of the tripeptides Ile-Pro-Pro and Val-Pro-Pro in more detail by using experimental animal models and hypertensive subjects.

The specific aims were:

1. *Experimental:* To investigate the antihypertensive mechanisms of Ile-Pro-Pro and Val-Pro-Pro and to evaluate the absorption, tissue distribution and excretion of Ile-Pro-Pro (I, II).
2. *Clinical:* To verify the long-term blood pressure lowering effects of peptide milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptides using different blood pressure measurement techniques and to evaluate the effects of a peptide milk containing Ile-Pro-Pro and Val-Pro-Pro on arterial stiffness (III, IV, V, VI).

4 MATERIALS AND METHODS

4.1 EXPERIMENTAL ANIMALS AND STUDY DESIGNS

Male spontaneously hypertensive rats (SHR; Harlan Sprague-Dawley, Indianapolis, IN, USA) were used in Study I and male Sprague-Dawley rats (Charles River Laboratories Ltd, Edinburgh, UK) in Study II. In unpublished data, the male double transgenic rats (dTGR) harbouring human renin and human angiotensinogen genes (Biotechnology and Animal Breeding Division, Füllinsdorf, Switzerland) were used (Jauhiainen et al. unpublished data). The rats were housed five to a cage (Study I, Jauhiainen et al. unpublished data) or in individual cages (Study II) in a standard experimental animal laboratory with automatic control of light cycles and temperature (light hours were 6.00/7.00 a.m. to 6.00/7.00 p.m., room temperature 18–24°C and humidity 40–60%). A standard laboratory diet of known formulation (SDS Rat and Mouse Maintenance Diet No. 1, 1 Stepfield, Witham, Essex, UK or 2018, Harlan Nederland, Horst, The Netherlands) and drinking fluid (tap water, mineral water, peptide water, peptide+mineral water or peptide milk) were available *ad libitum*. The consumption of feed was monitored weekly and drinking fluids daily throughout the study. The blood pressure was measured weekly by the same trained person with tail-cuff blood pressure measurement (IITC Life Science, Woodland Hills, CA, USA) (Study I, Jauhiainen et al. unpublished data). In all experimental studies, the rats were housed in metabolic cages. In Study I, 24-hour housing in metabolic cages took place at the beginning and end of the treatment period, and in Study II, 24-hour and 48-hour housing followed single administration of the peptide. The blood samples were taken at the end of the

treatment period (Study I, Jauhiainen et al. unpublished data) or at scheduled times up to 24 or 48 h post dosing (Study II).

The study protocol was approved by the Animal Experimentation Committee of the Institute of Biomedicine, Helsinki, Finland (Study I, Jauhiainen et al. unpublished data). Study II was performed under Home Office licence PPL 60/2687, Toxicology of Medical and Veterinary Materials, Edinburgh, UK (Table 5).

Table 5. *Experimental animals.*

	Strain	Number at the beginning of the treatment	Treatment period	Weight at the end of the treatment	Age
Study I	SHR	50	9 weeks	330–350 g	8–17 weeks
Study II	Sprague-Dawley	11	single administration	230–280 g	7–8 weeks
Unpublished data	dTGR	35	3 weeks	220–240 g	4–7 weeks

4.2 SUBJECTS AND STUDY DESIGNS

Study III

The long-term blood pressure lowering effects of *Lactobacillus helveticus* fermented milk containing Ile-Pro-Pro and Val-Pro-Pro (peptide milk, peptides 5 mg/d) were investigated in Study III. Thirty-nine hypertensive volunteers with a mean systolic blood pressure of ≥ 140 mmHg and/or mean diastolic blood pressure of ≥ 90 mmHg in office blood pressure measurements were included in the randomised, placebo-controlled, double-blind study. The subjects were randomly allocated to two groups to receive either the peptide milk containing Ile-Pro-Pro and Val-Pro-Pro or a placebo drink for 21 weeks after a two-week run-in period (Figure 5). Separate randomisation lists were used for those who were taking antihypertensive medication and those who were not. The blood pressure and heart rate were measured at home using a fully automatic blood pressure recorder (Omron M4, Omron Matsusaka Co Ltd, Matsusaka, Japan). Daily use of the study products was recorded by means of a pre-tested postal questionnaire. The subjects were also asked to report if they had experienced any adverse effects.

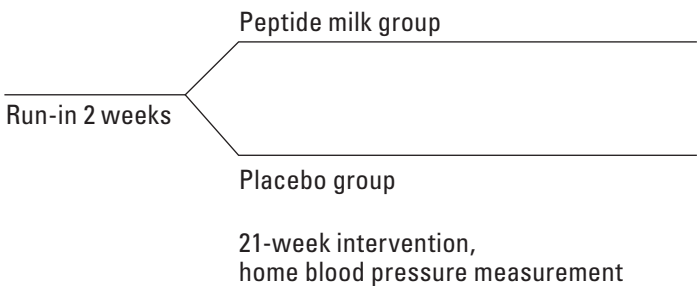


Figure 5. Study design in Study III.

Study IV

The effects of *Lactobacillus helveticus* fermented milk containing high concentrations of the tripeptides Ile-Pro-Pro and Val-Pro-Pro (peptide milk, peptides 50 mg/d) on blood pressure, together with any adverse effects, were examined in Study IV. Ninety-four hypertensive subjects with a mean systolic blood pressure of 140–180 mmHg and a diastolic blood pressure of 90–110 mmHg in office blood pressure measurements participated in this randomised, placebo-controlled, double-blind study. Exclusion criteria were blood pressure lowering medication, unstable coronary artery disease, diabetes mellitus, malignant diseases, alcohol abuse, milk allergy and pregnancy. The subjects were randomly allocated to two treatment groups (peptide milk group and placebo group) after a four-week run-in period. This was followed by a ten-week intervention period and a four-week follow-up period (Figure 6). Blood pressure was measured at the beginning and end of the ten-week intervention period with an automatic 24-hour blood pressure recorder (SpaceLab ABP 90207, Redmont, CA, USA). Subjects were asked to fill in a form about their daily use of the test products. The subjects were also asked, at every visit, whether they had experienced any adverse effects.

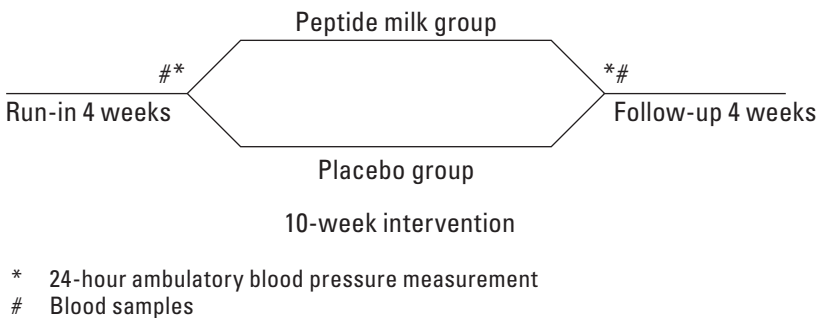


Figure 6. Study design in Studies IV and V.

Study V

Study V was analysed from the data of Study IV, so the study design (Figure 6.) and the inclusion criteria for subjects in Study V were the same as in Study IV. The analyses of ambulatory arterial stiffness index (AASI) included 94 subjects who participated in the 24-hour ABPM.

Study VI

The effect of *Lactobacillus helveticus* fermented milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptides (peptide milk) on vascular function was investigated in Study VI. Subjects with a systolic blood pressure of 140 to 155 mmHg and diastolic blood pressure of 85 to 99 mmHg in office and in 24-hour ABPM were included. Eighty-nine subjects participated in this double-blind, randomised, placebo-controlled study. After a four-week run-in period, the subjects were randomly allocated to an active group that received bioactive peptides or to a placebo group. During the first 12-week intervention period, the peptide dose was 5 mg/d (peptide milk 1); during the second 12-week intervention period, the peptide dose was 50 mg/d (peptide milk 2) in the peptide milk group. After the two 12-week intervention periods, there was a four-week follow-up period (Figure 7). Arterial stiffness and endothelial function testing was performed at the beginning and end of the intervention periods with pulse wave analysis (PWA) using a SphygmoCor (SphygmoCor, AtCor Medical, Sydney, Australia) device. Blood pressure was recorded at the same time points twice in the lying position (Omron M4, Omron Matsusaka Co. Ltd., Kyoto, Japan)]. Subjects were asked to fill in a form about their daily use of the test products and to report any adverse effects over the whole study period. Subjects were also asked at every study visit about adverse effects. Demographic characteristics of the study subjects are presented in Table 6.

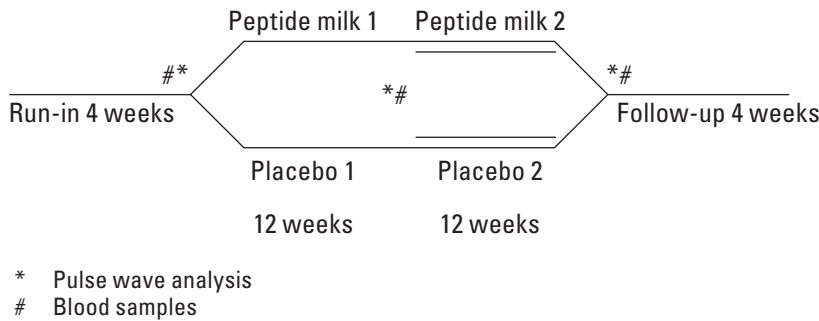


Figure 7. Study design in Study VI.

Ethics

The clinical studies were conducted according to the Helsinki Declaration and Good Clinical Practice. The Ethics Committee of the Department of Medicine, Tampere University Central Hospital (Studies IV and V), and the Ethics Committee of the Joint Authority for the Hospital District of Helsinki and Uusimaa (Study VI) approved the study protocols. All the subjects received both written and oral information regarding the trial and gave their written consent.

Table 6. Demographic characteristics of the study subjects.

Characteristics	Study III Home blood pressure measurement		Study IV 24-hour ABPM		Study V AASI		Study VI Office blood pressure measurement	
	Peptide milk N = 22	Placebo N = 17	Peptide milk N = 53	Placebo N = 55	Peptide milk N = 47	Placebo N = 47	Peptide milk N = 45	Placebo N = 44
Age, years , mean (SD)	51 (7)	48 (7)	51 (12)	51 (12)	51 (12)	53 (11)	49 (5)	49 (5)
Systolic blood pressure, mmHg, mean (SD)	152 (13)	149 (13)	148 (8.1)	151 (8.3)	133 (9.9)	130 (9.6)	155 (13.9)	151 (14.8)
Diastolic blood pressure, mmHg, mean (SD)	96 (6)	95 (6)	94 (6.2)	93 (6.1)	83 (8.0)	80 (7.0)	94 (8.8)	95 (12.2)
BMI, kg/m ² /weight, mean (SD)	*	**	28.6 (5.5)	28.6 (5.5)	29.0 (5.6)	28.5 (4.4)	27.6 (3.6)	28.5 (3.9)
Number of males/females	10/12	9/8	19/34	21/34	29 (62)	33 (70)	27/18	27/17
Treatment period, weeks	21		10		10		12+12	
* weight = 85.6 (16) kg	ABPM = ambulatory blood pressure measurement							
** weight = 77.6 (17) kg	AASI = ambulatory arterial stiffness index							

4.3 MEASUREMENT OF BLOOD PRESSURE

■ Experimental studies

4.3.1 Tail-cuff method (Study I, Jauhainen et al. unpublished data)

In the studies with SHR and dTGR the effect of different treatments on systolic blood pressure was investigated using a tail-cuff blood pressure analyser (IITC Life Science, Woodland Hills, CA, USA). The systolic blood pressure and heart rate of each rat were monitored weekly. Before measurement, the rats were warmed at +32°C for 15 minutes to improve detection of the pulse of the tail artery. The systolic blood pressure value was defined as the mean of three successful measurements without signal disturbances.

■ Clinical studies

4.3.2 Office blood pressure measurement (Study VI)

Office blood pressure was measured using a fully automatic blood pressure recorder (Omron M4, Omron Matsusaka Co. Ltd., Kyoto, Japan). Blood pressure was measured after a 15-minute rest in the lying position in the morning (8–10 a.m.). If the difference between the first two systolic blood pressure measurements was more than 5 mmHg, further measurements were made. If more than two measurements were made, those measurements between which the difference in systolic blood pressure was less than 5 mmHg were included in the analysis. The subjects were asked not to eat or drink coffee for 12 hours before the blood pressure measurement and to avoid heavy exercise a day before the measurement.

4.3.3 Home blood pressure measurement (Study III)

In the long-term clinical trial, blood pressure on the first study visit was measured from the left arm by a nurse using a fully automatic blood pressure recorder (Omron M4, Omron Matsusaka Co. Ltd., Kyoto, Japan). Blood pressure was measured in a sitting position after a 10-minute rest. At the second visit, the subjects were taught how to measure their own blood pressure, following which they measured their own blood pressure at home on the same weekday every week, in the morning, one hour after waking. The subjects were asked not to eat, smoke, exercise or take antihypertensive medication before the blood pressure measurement. The inclusion of blood pressure values in the analysis was determined using the same criteria as described earlier (see 4.3.2).

4.3.4 Twenty-four-hour ambulatory blood pressure measurement (Study IV)

In Study IV blood pressure was measured at the beginning and end of the ten-week intervention period with an automatic 24-hour blood pressure recorder (SpaceLab ABP 90207, Redmont, CA, USA). Blood pressure was measured four times an hour during the daytime and twice an hour during the night. During the day the measurements were made after a signal and during the night time silently. The measurement was accepted if at least 80% of the readings were successful, otherwise the measurement was repeated.

4.4 ARTERIAL RESPONSES

■ Experimental studies

4.4.1 Arterial preparations and responses (Study I)

Immediately after decapitation, the mesenteric artery and abdominal aorta of the SHR were cleaned of adherent connective tissue. The mesenteric artery and aorta rings (3 mm long) were placed between stainless steel hooks and suspended in an organ bath chamber in Krebs-Ringer buffer (pH 7.4). The rings were equilibrated for 45 minutes at + 37°C with a resting tension of 1.0 g. The force of contraction was recorded with a polygraph (FTO3 transducer, Model 7P122E Polygraph, Grass Instrument Co, Quincy, MA, USA). The presence of functional endothelium was confirmed by observing the relaxation response to acetylcholine after noradrenaline contraction in the arterial rings. The cumulative relaxation responses to acetylcholine (endothelium-dependent relaxation) and to sodium nitroprusside (endothelium-independent relaxation) were measured after noradrenaline precontraction.

4.4.2 ACE-inhibitory activity (Study I, Jauhiainen et al. unpublished data)

The ACE-inhibitory activity of Ile-Pro-Pro and Val-Pro-Pro *in vitro* was measured by preincubating the arterial rings from normotensive Sprague-Dawley rats with tripeptides Ile-Pro-Pro and Val-Pro-Pro for 10 minutes and measuring the response to a single administration of Ang I (1 µM)(Jauhiainen et al. unpublished data). The contraction response to Ang I (0.1 µM) in the mesenteric artery rings was also measured in Study I. The force of contraction was recorded with a polygraph (FTO3 transducer, Model 7P122E Polygraph, Grass Instrument Co, Quincy, MA, USA).

■ Clinical studies

4.4.3 Ambulatory arterial stiffness index (Study V)

The AASI was calculated from the ambulatory blood pressure readings of Study IV. The regression slope of diastolic blood pressure on systolic blood pressure was computed from the individual 24-hour blood pressure readings taken at 30-minute intervals from 8 a.m. to 10 p.m. and at 60-minute intervals from 10 p.m. to 8 a.m. AASI was defined as 1 minus the regression slope, as described by Li et al. (Li et al. 2006b).

4.4.4 Pulse wave analysis (Study VI)

Arterial stiffness was assessed by measuring of PWA using a SphygmoCor (SphygmoCor; AtCor Medical, Sydney, Australia) device. Blood pressure was recorded twice in the dominant arm in the lying position using a validated technique (Omron M4, Omron Matsusaka Co. Ltd., Kyoto, Japan). Radial artery waveforms were recorded with a high-fidelity micromanometer (SPC-301, Millar Instruments, Houston, TX, USA) from the wrist. The aortic augmentation index (AIx) was calculated as the augmentation of aortic systolic blood pressure by the reflected pulse wave, expressed as a percentage of the aortic pulse pressure. Because AIx is influenced by heart rate, AIx adjusted to a heart rate of 75 beats/min calculated by the software was used in the analysis (Wilkinson et al. 2000). The subjects were examined after a 12-hour fast and were asked to avoid alcohol consumption and exercise on the day prior to the measurements. The examinations were conducted in a quiet, temperature-controlled room after a 15-minute rest.

The time to return of the reflected wave (Tr) was calculated as the time from the beginning of the derived aortic systolic pressure waveform to the inflection point. Tr can be used as a substitute for pulse wave velocity (a higher pulse wave velocity will result in a shorter Tr) (London et al. 1992). Vascular endothelial function was studied using the PWA method developed by Wilkinson et al. (Wilkinson et al. 2002a). This method investigates the effects of nitroglycerin (endothelium-independent relaxation) (500 µg tablet, Nitro, Orion, Espoo, Finland) and salbutamol (endothelium-dependent relaxation) (2x200 µg inhalations of salbutamol, Ventoline Evohaler®, GlaxoSmithKline, London, UK) on the AIx.

4.5 COLLECTION OF SAMPLES

■ Experimental studies

Blood samples

In animal experiments the rats were rendered unconscious with CO₂/O₂ (95%/5%) (AGA, Riihimäki, Finland). Blood samples were collected after decapitation (Study I, Jauhiainen et al. unpublished data) or by cardiac puncture (Study II). Blood samples were analysed immediately (Study II) and/or taken into chilled tubes, centrifuged (3000 rpm, 20 minutes, +4°C) and stored at -80 °C until the biochemical determinations (Study I, II).

Tissues

In Study I the kidney and aorta were removed and snap-frozen in liquid nitrogen and stored at -80°C until the determinations. *In the unpublished data*, the heart and kidneys were removed and weighed, and the ratios to body weight were calculated. For morphological analysis, the renal samples were fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol, and embedded in paraffin. Sections of 2–3 µm were cut with a microtome and placed on glass. The sections were deparaffinised and rehydrated before staining with Masson's trichrome. Renal damage was analysed in a blind fashion and divided into four scores of 0–4 (0=normal and 4=severe damage) (Jauhiainen et al. unpublished data). *In Study II*, the tissues were collected and homogenised and duplicate samples were prepared for the determination of radioactivity levels.

Metabolic cages

Urine was collected using metabolic cages (24 h or 48 h) (Studies I, II and Jauhiainen et al. unpublished data). Urine volumes were measured and the samples were stored at -80°C until biochemical determinations. In Study II, faeces were also collected and the levels of radioactivity were determined. In Study I, the consumption of feed and drinking fluids was measured and the intake of minerals and peptides was calculated.

■ Clinical studies

In clinical studies (Studies IV and VI), blood samples were taken from the cubital vein at the beginning and end of the intervention phase after an overnight (12-hour) fast. The blood samples were stored at -80°C until the determination of serum total, LDL and HDL cholesterol and triglycerides, ACE activity, C-reactive protein and safety markers (blood cell count, serum creatinine, urate and gamma glutamyl transferase).

4.6 BIOCHEMICAL DETERMINATIONS

4.6.1 Peptide and mineral concentrations (Studies I, III, IV, VI)

The peptide concentrations of the fermented milk products were determined by the method of Masuda et al. (Masuda et al. 1996). The peptide fraction was obtained by gel filtration chromatography (Superdex Peptide HR 10/30, Amersham Pharmacia Biotech, Bucks, UK) and analysed by reversed-phase High Performance Liquid Chromatography (HPLC) (Novapak C18, Waters Alliance HPLC, Milford, MA, USA). Minerals were determined using an Elan 6100 ICP-MS (Inductively Coupled Plasma Mass Spectrometer) (PerkinElmer SCIEX, Shelton, USA). For the calcium analysis, the samples were weighed and transferred to a muffle furnace (NaberthermN11, Bremen, Germany) and maintained at +550°C overnight until a white ash was obtained. The samples were cooled and dissolved in nitric acid and analysed by ICP-MS.

4.6.2 Plasma and serum analyses (Studies I, IV, VI)

In the experimental study, plasma renin activity (PRA) was measured using a radioimmunoassay of Ang I (Medix Angiotensin I test®, Medix Biochemica, Kauniainen, Finland).

In the clinical studies serum total cholesterol, HDL cholesterol and triglycerides were measured enzymatically. LDL cholesterol was calculated using the Friedewald equation (Friedewald et al. 1972). Serum ACE activity was determined spectrophotometrically using the artificial substrate method (Groff et al. 1993), and sensitive C-reactive protein was determined from serum using a turbidimetric immunoassay (Wako Chemicals, Neuss, Germany).

4.6.3 Urine analyses (Study I)

Urinary sodium and potassium were determined by flame emission spectrometry (Burtis et al. 1975) and calcium and magnesium by flame atomic absorption spectrometry (Cali et al. 1973). Urinary creatinine was determined enzymatically and phosphate by spectrometry. Urinary nitrate and nitrite were determined using the Griess reaction (Green et al. 1982).

4.6.4 Radioactivity analyses (Study II)

The samples (whole blood, faeces and tissue) were subjected to liquid scintillation counting using a Packard TR 2100 Liquid Scintillation Analyser (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA) with automatic quench correction by an external method. All samples analysed by liquid scintillation counting for this

study were above the reliable quantification limits. Plasma samples were analysed by means of HPLC (HP 1100 Series, Agilent Technologies, Inc., Santa Clara, CA, USA) and by size-exclusion chromatography. Plasma samples were injected directly onto the column alongside the protein molecular weight standard solutions and the Ile-Pro-[^{14}C]Pro standard solution. The molecular mass of each of the resolved radioactive peaks was estimated using the calibration curve. Plasma samples were taken for further analyses by reversed-phase HPLC to identify the radioactive compounds present. Liquid chromatography-mass spectrometry (LC-MS) analysis was then performed on the reference standards and isolated peaks of interest to confirm that Ile-Pro-Pro was present in the sample.

4.6.5 Plasma protein binding properties (Study II)

The plasma protein binding properties of Ile-Pro-Pro and Pro-Pro were examined *in vitro* by incubating the peptides with human serum albumin and with human plasma. The incubations were performed at +37°C for 30 minutes in 4% human serum albumin (Sigma-Aldrich, St. Louis, MO, USA) and in plasma with a peptide concentration of 10 μM Ile-Pro-Pro or 10 μM Pro-Pro. After incubation, the samples were deproteinised by ultrafiltration with centrifugal devices of 10 kD cut-off (Microsep Omega® 10 K, Pall Corporation, East Hills, NY, USA), for two hours, at 3000 x g at room temperature.

4.7 PRODUCTS

The *Lactobacillus helveticus* fermented milk product containing Ile-Pro-Pro and Val-Pro-Pro tripeptides (peptide milk) was produced by Valio Ltd, Helsinki, Finland, from heat-treated, low-lactose skimmed milk inoculated with single-strain *Lactobacillus helveticus* (LBK 16 H strain) culture under aseptic conditions. The milk was fermented for 18–20 h in optimal growth conditions in order to reach a high proteolytic activity at +37°C to a final pH of 4.0–4.2. In studies with fermented milk containing high concentrations (dose 50 mg/d) of Ile-Pro-Pro and Val-Pro-Pro, the *Lactobacillus helveticus* fermented milk was enriched with peptide concentrate. The Ile-Pro-Pro and Val-Pro-Pro (Bachem AG, Bubendorf, Switzerland) in drinking fluid were dissolved in tap water. In mineral water, the minerals calcium (calcium lactate gluconate), potassium (potassium citrate) and magnesium (magnesium chloride) were dissolved in boiled tap water. The daily doses of peptides and minerals are shown in *Table 7*.

Table 7. Daily dose of peptides and minerals

	Peptide milk	Peptide water	Peptide and mineral water	Mineral water	Radiolabelled IPP
Experimental studies, mg/kg of body weight					
Study I	IPP 2.1 and VPP 2.1, Na 38, K 384, Ca 256, Mg 21	IPP 1.4 and VPP 1.4	IPP 1.6 and VPP 1.6, Na 38, K 278, Ca 221, Mg 20	Na 36, K 263, Ca 209, Mg 19	
Study II					7 mg/kg, radiolabelled IPP was 2.2-2.5%, the rest unlabelled IPP
Clinical studies, daily dose mg					
Study III	IPP 2.25 and VPP 3, Na 48, K 465, Ca 300, Mg 26				
Study IV	IPP 22.5 and VPP 30, Na 123, K 1530, Ca 690, Mg 93				
Study V	IPP 22.5 and VPP 30, Na 123, K 1530, Ca 690, Mg 93				
Study VI	Peptide milk 1: IPP 3.4 and VPP 3.7, Na 69, K 480, Ca 310, Mg 26 Peptide milk 2: IPP 23.2 and VPP 26.4, Na 128, K 1000, Ca 760, Mg 72				
IPP = Ile-Pro-Pro VPP = Val-I-Pro-Pro					

4.8 STATISTICAL ANALYSIS

The results are expressed as means with standard deviations (SD) or with standard errors of the mean (SEM). The most important outcomes are reported with 95% confidence intervals (CI). The changes within treatment groups were analysed using the paired t-test. The differences in changes between treatment groups were compared using either a t-test for independent samples or analysis of covariance (ANCOVA) with covariates. In the case of longitudinal data, analysis of variance for repeated measurements was performed to compare the results of different treatment groups. SPSS (version 11.5; SPSS Inc, Chicago, IL, USA) and STATA (version 9.2; StataCorp, College Station TX, USA) were used for the statistical analyses.

5 RESULTS

5.1 EFFECT OF PEPTIDE MILK ON BLOOD PRESSURE

■ Experimental studies (Study I and Jauhiainen et al. unpublished data)

Peptide milk tended to attenuate the development of hypertension in SHR and attenuated the development of hypertension in dTGR. At the end of the nine-week treatment period (Study I), the systolic blood pressure in the peptide milk group was -16.9 mmHg ($p=0.053$) lower than in the control group. This finding was supported by unpublished data on dTGR, where the mean blood pressure value in the peptide milk group ($n=10$) was 173.5 mmHg during the three-week treatment period while that in the control group ($n=15$) was 192.2 mmHg, the difference between the groups being about -19.0 mmHg ($p=0.023$) (Jauhiainen et al. unpublished data). In SHR, a slight, non-significant, effect on blood pressure was also seen with pure tripeptides in water (-8.3 mmHg vs. water control, $p=0.379$) and with minerals (Ca, K, Mg) and tripeptides in water (-12.7 mmHg vs. water control, $p=0.153$). The beneficial effect on blood pressure with pure tripeptides was not seen in dTGR (*Figure 8*).

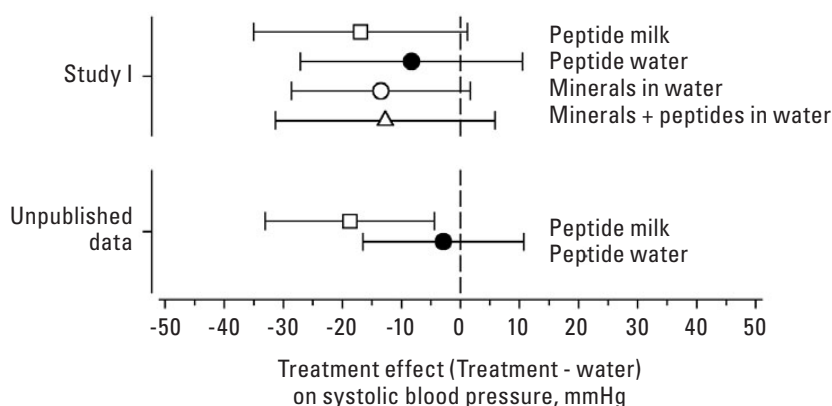


Figure 8. Treatment effects on systolic blood pressures in SHR (Study I) and in dTGR (Jauhiainen et al. unpublished data), mean \pm SD.

■ Clinical trials (Study III, IV, VI)

Baseline blood pressure values were measured using office blood pressure measurement (Study III, IV, VI). In the whole study group in Study III, the baseline values (mean \pm SD) for systolic blood pressure were 151 ± 13 mmHg and those for diastolic blood pressure 96 ± 6 mmHg. In Study IV, the systolic blood pressure values in repeated office blood pressure measurements were 149 ± 8 mmHg and diastolic blood pressure values 93 ± 6 mmHg. In Study VI, the baseline systolic blood pressure values were 153 ± 14 mmHg and diastolic blood pressure values 95 ± 11 mmHg.

Systolic blood pressure tended to decrease more in the peptide milk group than in the placebo group. In Study III, the treatment effect was significant only in per protocol analysis [-6.7 mmHg (95% CI -12.8 to -0.7), $p=0.030$], but not in intention-to-treat analysis. In Study IV, the treatment effect was significant [-4.1 mmHg (95% CI -6.6 to -1.8), $p<0.001$]. In Study VI, a high dose of peptides lowered systolic blood pressure compared to the baseline level [-4.6 mmHg (95% CI -8.4 to -0.8), $p=0.018$], but the treatment effect was not significant.

Diastolic blood pressure tended to decrease in Study III, but the treatment effect was not significant even in the per protocol analysis [-3.6 mmHg (95% CI -7.4 to 0.1), $p=0.059$]. In Study IV, diastolic blood pressure decreased more in the peptide milk group than in the placebo group and the treatment effect was significant [-1.8 mmHg (95% CI -3.7 to 0.0), $p=0.048$]. In Study VI, with a high dose of peptides, the diastolic blood pressure decreased compared to the baseline level [-3.7 mmHg (95% CI -6.1 to -1.3), $p=0.004$], but the treatment effect was not significant (Figure 9).

In Study VI, no significant changes in systolic or diastolic blood pressure were observed after the first 12-week intervention with low-dose peptide treatment.

In Study III, in the peptide milk group systolic and diastolic blood pressure fell by -17.7 ± 5.4 mmHg and -9.8 ± 5.6 mmHg, respectively, for subjects without medication ($n=13$) and by -16.1 ± 14.4 mmHg and -8.9 ± 8.4 mmHg for subjects with medication ($n=9$). In the placebo group systolic and diastolic blood pressure fell by -11.3 ± 14.0 mmHg and -4.3 ± 8.5 mmHg, respectively, for subjects without medication ($n=10$), and by -11.1 ± 18.4 mmHg and -8.8 ± 8.8 mmHg for subjects with medication ($n=7$). None of these results were significant.

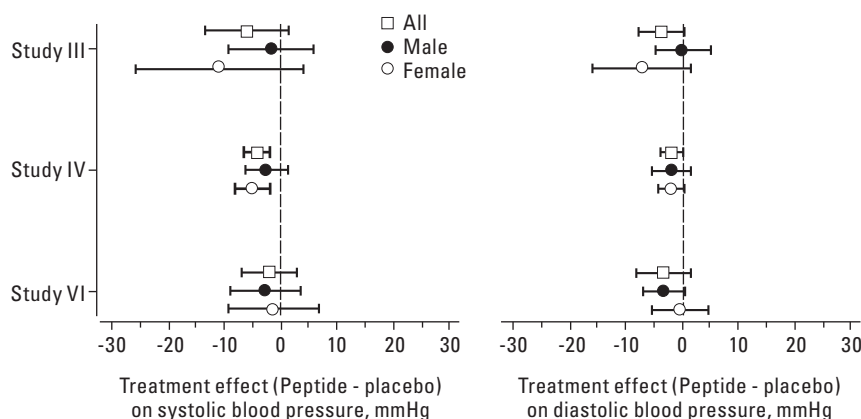


Figure 9. Treatment effects on systolic and diastolic blood pressures in Studies III, IV and VI, mean \pm SD.

5.2 EFFECT OF PEPTIDE MILK ON VASCULAR FUNCTION

■ Experimental study (Study I)

The most marked, maximal, cumulative relaxation response *in vitro* in the mesenteric artery and aorta in endothelium-dependent relaxation to acetylcholine was seen in the mineral + peptide group ($p=0.023$). No differences between the treatment groups were seen in the endothelium-independent relaxations to sodium nitroprusside.

■ Clinical studies (Study V, VI)

Ambulatory arterial stiffness index (Study V)

During the 10-week intervention period, AASI improved significantly in the peptide milk group, but not in the placebo group (*Table 8*). The improvement in AASI was 12% in the peptide milk group and 5% in the placebo group as compared to baseline levels. There were no significant differences between the study groups in the AASI ($p=0.40$).

Table 8. Changes in AASI from baseline to the end of the 10-week treatment period.

	Baseline Mean (SD)	Change by week 10 Mean (95% CI)	p-value
Peptide milk, n=47	0.36 ± 0.15	-0.043 (-0.084 to -0.001)	0.043
Placebo, n=47	0.36 ± 0.17	-0.019 (-0.074 to 0.035)	0.47

Effect of peptide milk on arterial stiffness and endothelial function (Study VI)

AIx, as an indicator of improved vascular function, decreased significantly in the peptide milk group compared to the placebo group [peptide milk group -1.53% (95% CI -2.95 to -0.12), placebo group +1.20% (95% CI 0.09 to 2.32), $p=0.013$] (*Figure 10*). The improvement in arterial stiffness was more noticeable in males. After the 24-week treatment period, the AIx values were significantly lower in males in the peptide milk group compared with the placebo group [peptide milk group -2.30% (95% CI -4.31 to -0.28), placebo group +1.74% (95% CI 0.44 to 3.04), $p=0.004$]. There was no difference in the change of AIx in females between the peptide milk group [-0.39%, (95% CI -2.34 to 1.56)] and the placebo group [+0.35%, (95% CI -1.77 to 2.48), $p=0.90$].

The Tr value did not differ between the groups. However, when comparing males, there was a difference between the groups [peptide group +1.7 ms (95% CI: -0.44 to 3.9), placebo group -2.1 ms (95% CI -4.1 to -0.1), $p=0.022$]. In women, the difference was not seen [peptide group -1.4 ms (95% CI -3.7 to 0.9), placebo group -1.6 ms (95% CI -5.2 to 1.9), $p=0.58$].

No differences between the groups were seen in the response to nitroglycerin-induced endothelium-independent relaxation [peptide group +0.33% (95% CI: -1.31 to 1.98), placebo group +1.20% (95% CI -0.74 to -3.15), $p=0.79$]. The same applies to the response to salbutamol-induced endothelium-dependent relaxation [peptide group -0.64% (95% CI: -2.41 to 1.12), placebo group +0.82% (95% CI -1.12 to 2.75), $p=0.61$] after 24 weeks' treatment.

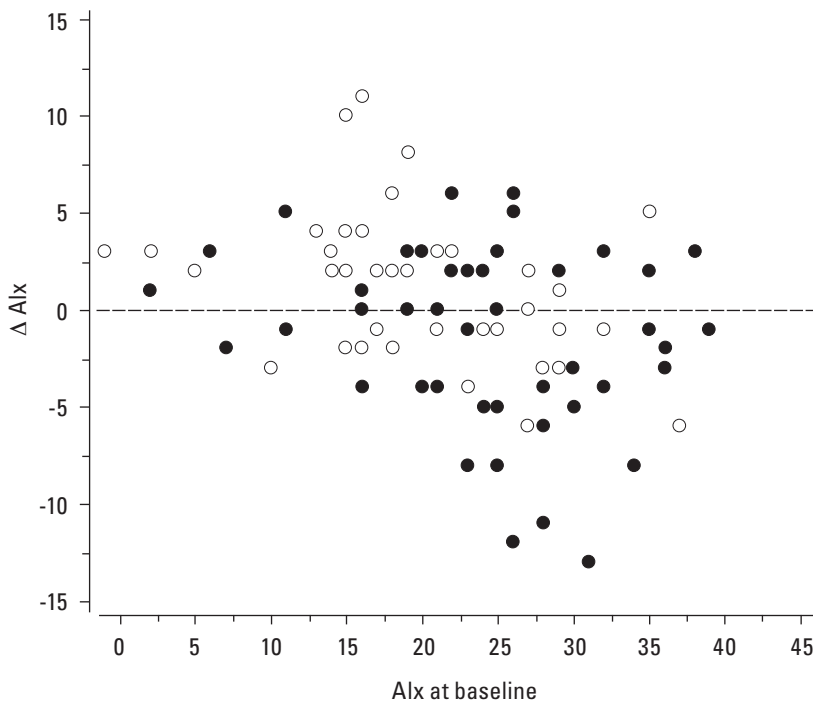


Figure 10. Alx values at the baseline before nitroglycerin and salbutamol administration and the change from baseline to 24 weeks (Δ Alx). ● = peptide milk $n=45$, ○ = placebo $n=44$.

5.3 BLOOD PRESSURE LOWERING MECHANISMS

■ Experimental studies

In Study I, the role of ACE inhibition as the mechanism by which Ile-Pro-Pro and Val-Pro-Pro lower blood pressure was investigated in SHR by measuring the plasma renin activity (PRA). The PRA of the peptide water group was lower than in the control group ($p=0.042$).

In *in vitro* analysis, the contraction response to Ang I in the mesenteric artery rings tended to attenuate in SHR receiving the peptide milk (Study I) and in arterial rings from Sprague-Dawley rats preincubated with pure tripeptides (Jauhiainen et al. unpublished data).

Blood pressure lowering mechanisms were also studied using dTGR, which develop malignant hypertension due to increased Ang II formation (Jauhiainen et al. unpublished data). Peptide milk showed a clear attenuation in blood pressure values, but neither the peptide milk nor peptide water showed any signs of reduced Ang II production. There were no differences between the groups in Ang II-induced end-organ damage measured by renal morphology. The damage score was 3.3 ± 0.2 in the dTGR control group ($n=15$), 3.0 ± 0.3 in the peptide milk group ($n=10$) and 2.8 ± 0.2 in the peptide water group ($n=10$) ($p=0.53$).

■ Clinical studies

In Study VI, the blood pressure lowering mechanism of peptide milk was evaluated by measuring arterial stiffness. In this study, pulse wave reflection induced by β_2 -adrenergic stimulation was not markedly improved [peptide milk group -0.64 (95% CI: -2.41 to 1.12), placebo group 0.82 (95% CI: -1.12 to 2.75), $p=0.61$]. This suggests indirectly that improved endothelial NO release capacity is not the mechanism by which peptide milk mediates its favourable circulatory effects.

5.4 KINETICS OF ILE-PRO-PRO PEPTIDE

■ Radioactivity analyses

In Study II, after a single intravenous administration of Ile-Pro- $^{[14]}\text{C}$ Pro, the mean blood concentration observed at 1 minute post-dose was 16.95 $\mu\text{g equiv/g}$. The highest blood concentration after a single oral administration of Ile-Pro- $^{[14]}\text{C}$ Pro was observed at two hours post-dose (6.79 $\mu\text{g equiv/g}$). The concentration of total radioactivity declined slowly after oral absorption and at 48 h was still 2.94 $\mu\text{g equiv/g}$.

Excretion of radiolabelled Ile-Pro-Pro was measured from urine and faeces. A notable level of radioactivity was still present in the residual carcass and whole tissues collected at 48 h post-dose. Radioactivity was distributed in the body after oral administration of Ile-Pro- $^{[14]}\text{C}$ Pro. The highest concentrations of total radioactivity were observed in most tissues at 4 h post-dose, with considerable levels of radioactivity observed in the bone marrow, adrenals and aorta as well as in the liver, kidneys and spleen. Chromatographic and mass spectrometric analysis of plasma samples proved that Ile-Pro- $^{[14]}\text{C}$ Pro was present in each sample analysed because the component detected at *ca.* 12 minutes during size-exclusion chromatography experiments was shown by mass spectrometry analysis to be Ile-Pro-Pro.

■ Plasma protein-binding properties

Ile-Pro-Pro at a concentration of 10 μM was incubated in 4% human serum albumin and human plasma for 30 minutes. After ultrafiltration, the concentrations of Ile-Pro-Pro found in the permeates were 100%. Pro-Pro concentrations in the permeates were 51% in human serum albumin and about 2% in human plasma after incubation and ultrafiltration. The *in vitro* tests thus showed that Pro-Pro attaches to plasma proteins (over 98%) whereas Ile-Pro-Pro is not bound.

6 DISCUSSION

Earlier experimental studies have shown that the bioactive peptides Ile-Pro-Pro and Val-Pro-Pro derived from milk casein have beneficial effects on blood pressure, while peptide milk has been shown to reduce blood pressure in clinical studies. However, the antihypertensive mechanisms, kinetics, long-term antihypertensive effects and the effects on vascular function in clinical use are not well known. This study investigated the blood pressure lowering effects and the effects on arterial stiffness of peptide milk in hypertensive subjects. The blood pressure lowering mechanisms of tripeptides Ile-Pro-Pro and Val-Pro-Pro and peptide milk, and the kinetics of Ile-Pro-Pro were investigated in these experimental studies.

6.1 METHODOLOGICAL ASPECTS

■ Experimental studies

Animal models

In the experimental studies, three different animal models were used to evaluate the blood pressure lowering mechanisms of Ile-Pro-Pro and Val-Pro-Pro and the kinetics of Ile-Pro-Pro. SHR is the most commonly used animal model for human essential hypertension. The animal model develops hypertension and its complications with increasing age. Even though this animal model is widely used, it has been criticised as not an appropriate model for human essential hypertension (for review, see Zicha and Kunes 1999).

The other animal model used in the unpublished data, dTGR, is suitable for studying RAS-related mechanisms, the effects of ACE inhibitors, AT₁ receptor

blockers and Ang II-induced pathogenesis (Bohlender et al. 1997). This animal model develops severe hypertension and lethal cardiac and renal end-organ damage at 7 weeks of age (Luft et al. 1999). The effects of nutritional factors in this animal model have been evaluated in only a few studies. Magnesium supplementation and lipoic acid have been shown to have beneficial effects after three weeks' treatment (Mervaala et al. 2003; Finckenberg et al. 2005). This model makes it possible to evaluate only rather short-term effects due to the malignancy of hypertension, whereas the treatment period in studies evaluating the effects of lifestyle modification on health should usually be relatively long. When the kinetics of a certain compound are investigated, the animal models used are usually healthy models. In the present study, Sprague-Dawley rats were used.

Blood pressure measurement technique

Blood pressure was measured using the tail-cuff method. This method is relatively simple and suitable for a large number of animals as in the present study, and it has shown to have a good correlation with direct intra-arterial recordings. However, the tail-cuff method may overestimate blood pressure levels because of the stress caused by the environment. Furthermore, only systolic blood pressure values can be registered (Plehm et al. 2006). The overestimation of the blood pressure level can be minimised, if the same person measures the blood pressure during the whole study period and if the surroundings are quiet. These factors were taken into account in the present study. The rats were kept at +32°C for 15 minutes before the measurement to obtain reliable blood pressure values. However, this temperature also represents a stressful situation for rats and might raise their blood pressure (Kenney et al. 1995). For this reason, the duration of exposure to this temperature was kept exactly the same for each rat so this possible effect on blood pressure was the same in all the treatment groups.

Arterial responses

It has been shown in experimental and clinical trials that aging and hypertension interact and are associated with long-term changes in arterial structure and function. Injuries to relatively large arteries can cause the major complications of hypertension (for review, see Safar et al. 1998). It is therefore important to examine the effects of antihypertensive compounds on arterial function (Safar et al. 2001; Izzo and Mitchell 2007). In the present study, we used the mesenteric artery and aorta as models to evaluate the effects of blood pressure lowering interventions with Ile-Pro-Pro and Val-Pro-Pro and peptide milk on the functioning of these arteries. The mesenteric artery can be used as a model of a resistance artery and is suitable

for use in studies measuring precontractions and reproducible relaxation responses. The aorta, on the other hand, can be used as a model of a conduit artery.

■ Clinical studies

Study designs and products

All the clinical studies were carried out as randomised, double-blind, and placebo-controlled trials. The studies contained a run-in period before the intervention period. The intervention periods, 10 to 24 weeks, were considered long enough for a significant reduction in blood pressure levels, because in earlier studies with Ile-Pro-Pro and Val-Pro-Pro peptide milk, a reduction in blood pressure was seen after an eight-week treatment period (Hata et al. 1996; Seppo et al. 2002). No earlier clinical references to the effects of peptide milk containing Ile-Pro-Pro and Val-Pro-Pro on vascular function were available.

The placebo product in all studies was similar in taste and appearance to the peptide milk. The placebo products were fermented milk drinks, just as the peptide milk. The subjects were well motivated and only about 3% withdrew from the studies after the run-in period. The subjects maintained normal diet and exercise habits throughout the study period, and the compliance for taking the study products daily was good in all studies.

In all the studies, blood pressure was slowly reduced when the treatment (including that with placebo) was started. This is a common phenomenon in blood pressure trials, which is why the run-in period at the beginning of the study is important (Asmar et al. 2001).

Blood pressure measurement techniques

Blood pressure was measured using three different techniques: office blood pressure measurement, home blood pressure registration and 24-hour ABPM. The office blood pressure measurement involves the biggest risk of variations and high blood pressure values, because even if all guidelines are followed, there is still variation. The prevalence of white coat hypertension in hypertension patients (office measurement) has been found to be approximately 20% (Pickering et al. 1988; Verdecchia et al. 1995). For subjects with white coat hypertension, better blood pressure readings are obtained with home blood pressure measurement and 24-hour ABPM. Home blood pressure measurements in hypertensive subjects have been reported to have high reproducibility and low placebo effect (Imai et al. 2001). The 24-hour ABPM is the gold standard and also covers the circadian rhythm (Giles 2006).

In the present study, a clear difference was shown between the office blood pressure measurement and the 24-hour ABPM. The average office baseline systolic blood pressure values were over 15 mmHg higher and diastolic blood pressure values were over 10 mmHg higher than the ambulatory values. The subjects in the office blood pressure measurement were mildly hypertensive (systolic blood pressure about 150 mmHg and diastolic 93 mmHg), but when blood pressure was measured with the ABPM, the blood pressure fell and the subjects were nearly normotensive (systolic blood pressure about 130 mmHg and diastolic 80 mmHg). This can be explained by the diurnal variation seen in ABPM, which includes, with healthy subjects, lower blood pressure values during the night (O'Brien et al. 2003).

Arterial responses

The effects of peptide milk on arterial stiffness were evaluated using a non-invasive PWA. In this analysis, the speed of the pulse going through arteries increases with increasing stiffness (Oliver and Webb 2003). When arterial stiffness is measured, the Clinical Applications of Arterial Stiffness advise that the subjects should not smoke or eat for at least three hours before the measurements and that they should have a 10-minute period of rest in a quiet room before the actual measurements (Van Bortel et al. 2002). These guidelines were taken strictly into account in the present study.

When arterial stiffness is measured using PWA, different components of the waveform, such as the AIx, can be derived as markers of arterial stiffness, and the reproducibility of the markers is generally reported to be good (Wilkinson et al. 1998). The AIx was calculated as the difference between the first and second systolic peaks, expressed as a percentage of the central pulse pressure, and can be used as a measure of arterial stiffness. Increased aortic and carotid AIx have been shown to be an independent predictor of cardiovascular disease risk, mortality and morbidity (London et al. 2001; Kingwell et al. 2002; Nurnberger et al. 2002). Recent studies show that AIx in the radial artery, which was measured in the present study, is another independent risk marker for cardiovascular diseases and provides information equivalent to the carotid arterial AIx (Weber et al. 2004; Sugawara et al. 2007).

The AASI is a novel index of vascular stiffness. AASI has been shown to correlate with aortic pulse wave velocity and with the central and peripheral systolic augmentation indexes (Li et al. 2006b). AASI has been shown to be an independent predictor of cardiovascular mortality and to be associated with organ damage in hypertensive patients (Leoncini et al. 2006; Li et al. 2006a). The measurement of AASI has also been criticised, and according to Westerhof et al. (2007), AASI

correlates with indicators of arterial stiffness but is not a measure of arterial stiffness (Westerhof et al. 2007). In the present study, the AASI values were within normal range according to the reported age-related AASI values, which are under 0.50 at 20 years and under 0.70 at 80 years (Li et al. 2006b).

6.2 EFFECTS OF PEPTIDE MILK ON BLOOD PRESSURE AND VASCULAR FUNCTION

BLOOD PRESSURE

■ Experimental studies

Peptide milk attenuated the development of hypertension in dTGR and tended to attenuate it in SHR. A similar but less potent tendency was seen with pure tripeptides and tripeptides in combination with minerals (Ca, K, Mg) in SHR. The effects on blood pressure of peptide milk and the pure tripeptides Ile-Pro-Pro and Val-Pro-Pro have been shown in earlier experimental trials by our group with SHR (Sipola et al. 2001; Sipola et al. 2002a). The long-term effects and the differences between the peptide milk group and the control group were seen after five to eight weeks' treatment (Nakamura et al. 1995b; Sipola et al. 2001; Sipola et al. 2002a); on the other hand, the effects of tripeptides have also been seen after a single oral administration (Nakamura et al. 1995b; Sipola et al. 2001; Sipola et al. 2002a). In the present study with SHR, the treatment period can therefore be considered long enough, even though significant differences could not be seen. In the study with dTGR, the treatment period was only three weeks because of the malignancy of hypertension and death of the animals, and therefore, the effects of peptide water on blood pressure may not have been seen during this treatment period.

The present study with SHR suggests that minerals may have an important role as blood pressure lowering components in peptide milk. The same tendency for attenuating the blood pressure values was seen with minerals as with pure tripeptides in water. In the study with SHR and dTGR, the peptide milk was the most effective. It tended to attenuate the development of hypertension in SHR and attenuated the development of blood pressure in dTGR. It is therefore suggested that peptide milk might also contain some other components that affect blood pressure. The effective components, such as minerals, might also be absorbed better from milk than from water (Kitts et al. 1992; Narva et al. 2004). The diet of the rats receiving peptide milk, mineral water or mineral+peptide water contained

about 0.6% more calcium and about 0.7% more potassium than the control diet because of the calcium and potassium contents of the drinking fluids. However, in earlier studies, the calcium supplementation showing attenuation in blood pressure values in SHR was 2 to 3% and that of potassium 3.5% (Pörsti 1992; Tolvanen et al. 1998). On the other hand, the blood pressure reductions observed in these studies were also more marked than in the present study. Therefore, the present study appears to indicate that, together with peptides, minerals might play an important role in the attenuation of hypertension.

■ Clinical studies

The peptide milk tended to lower blood pressure in all clinical studies. The blood pressure lowering effect was studied with two daily doses (about 5 mg and 50 mg) of tripeptides. In earlier published clinical studies, the daily dose of different peptides or proteins has varied from 2.6 mg to 20000 mg (20g), but the blood pressure reduction did not seem to be related entirely to the peptide dose in these studies (*Table 4*). An interesting fact in these studies is that they report only positive results with milk peptides and proteins. This might be a bias in referring to this literature. However, because of these reported doses in the literature, it could be speculated that the peptide dose attains maximal efficacy with quite small doses and that higher doses do not give a better response, as is the case also with some other functional components such as plant stanols (Cater et al. 2005) and antihypertensive medication.

In the present study, the blood pressure lowering effect was seen with 50 mg of tripeptides, and a tendency for lowering blood pressure was also seen when the dose was 5 mg. In Study VI, the effects of both doses were evaluated and the effect was only seen with the high peptide dose. One explanation for this could be the relatively high baseline blood pressure values, since the blood pressure values as inclusion criteria were measured using 24-hour ABPM. Therefore it could be that the small peptide dose in this trial was not effective enough to give results. On the other hand, in this trial blood pressure was measured during the intervention by office blood pressure measurement, which is the technique with the highest variation, i.e. small reductions in blood pressure values may not have been observed.

At 10 to 24 weeks, the intervention periods in this study were relatively long compared with other studies with peptides. It is important to show that the blood pressure lowering effect is stable and lasts over a long period and that long-term use does not cause any adverse effects. Interestingly, it was shown in the present study that the blood pressure reduction response was more marked in women in Studies III and IV. Demographic characteristics cannot explain this difference.

The peptide milk contained more calcium, potassium and magnesium than the placebo products, and therefore some of the blood pressure lowering effect could be attributable to these minerals. However, minerals cannot fully explain the difference between the groups, because when comparing to the earlier studies, small reductions in blood pressure have been achieved with calcium supplementation of about 1200 mg/d, systolic blood pressure fell by about 2 mmHg and diastolic blood pressure by about 1 mmHg (van Mierlo et al. 2006) and with potassium supplementation of about 2900 mg/d systolic blood pressure fell by about 3 mmHg and diastolic blood pressure by about 2 mmHg (Whelton et al. 1997). In the present study, the subjects in the peptide milk group received 105–420 mg more calcium and 240–1080 mg more potassium than the placebo group.

VASCULAR FUNCTION

■ Experimental studies

In the experimental studies, an improvement in endothelium-dependent relaxation was seen with minerals+peptides in water. This is interesting because the peptide milk contained the same peptides and minerals. The intake of minerals was even slightly higher in the peptide milk group because of higher consumption. In addition, calcium absorption might be enhanced due to the caseinophosphopeptides in milk (Kitts et al. 1992; Narva et al. 2004). Minerals alone have been shown to have a positive effect on arterial responses. Calcium has been shown to enhance vasorelaxation in experimental trials (Mäkynen et al. 1995; Jolma et al. 2003; Kähönen et al. 2003). In the present study, the extra calcium intake in the peptide milk group compared to the control group was, however, much smaller than the intake of calcium in earlier studies (Mäkynen et al. 1995; Jolma et al. 2003; Kähönen et al. 2003). Potassium and magnesium also have beneficial effects on vascular function, and the positive effects of potassium might be mediated by increasing endothelial nitric oxide production (Mäkynen et al. 1995; Wu et al. 2006; Dorrance et al. 2007). In the present study, the potassium intake was also only about one third of the intake in the earlier studies. However, even though the intakes of calcium and potassium were smaller in the present study than in the earlier studies, they could partly explain the positive effect. ACE inhibitors have also been shown to improve relaxation responses in rats, and the slight ACE inhibitory activity of Ile-Pro-Pro and Val-Pro-Pro might therefore partly explain this result (Mervaala et al. 1998b; Baluchnejadmojarad et al. 2004).

■ Clinical studies

The clinical studies showed that peptide milk containing a high dose of tripeptides improves AIx and AASI in hypertensive subjects. This effect might be related to the improved sensitivity of vascular smooth muscle cells. In the present study, nitroglycerin administration was used to evaluate the endothelium-independent relaxation and salbutamol to evaluate the endothelium-dependent relaxation. Because no clear differences were seen between the groups after these measurements, the present study could not demonstrate that the clear vasodilation seen in the basal values with peptide milk is caused by the stimulation of endothelial NO release. However, because basal NO release by the endothelium is an important determinant of pulse wave reflection, it can be hypothesised that the peptide milk improves basal endothelial NO release or, on the other hand, PGI₂ or EDHF formation, although the maximal capacity of the release of these compounds remained unaffected. The results in Tr in males also support beneficial effects of peptide milk. The Tr value was slightly increased in the peptide group, and because Tr is a risk factor for coronary heart disease (Hayashi et al. 2002; Weber et al. 2004), this is an interesting finding.

It is unlikely that the 24-week intervention period could cause any major changes in the elastic properties of the arterial wall. The mechanism is more likely to be a reduction in arterial tone. This could reduce arterial stiffness as a result of decreasing tension in vascular smooth muscle (Wilkinson et al. 2000).

The reduction in AIx was only seen in males in this study. However, the same active treatment also produced a more marked reduction in blood pressure in males. AIx increases with age and has been shown to be higher in women (Segers et al. 2007). The differences in age cannot explain this difference between the genders because the age was similar between the active and placebo groups and between genders. Female gender, on the other hand, could play some role in this difference. Central arterial elastic properties vary throughout the menstrual cycle in women (Hayashi et al. 2006), and the cycle was not taken into consideration in the present study. However, it cannot be concluded from these results that peptide milk is effective only in males, because the results on blood pressure in two other clinical studies (Studies III and IV) were the opposite.

6.3 BLOOD PRESSURE LOWERING MECHANISMS AND KINETICS OF ILE-PRO-PRO AND VAL-PRO-PRO

Blood pressure lowering mechanisms

A tendency for ACE inhibitory activity was seen *in vitro* but not *in vivo*. Therefore, the present study does not support the earlier findings on ACE inhibition by Ile-Pro-Pro and Val-Pro-Pro. This might be partly explained by the use of a very severely hypertensive animal model, dTGR, in the experimental studies. It has earlier been shown that these tripeptides decrease ACE activity in the aorta after a long-term intake and even after a single oral administration (Nakamura et al. 1995b; Nakamura et al. 1996). It has also been shown in SHR that these tripeptides in fermented milk increase plasma renin activity, which is a marker of ACE inhibition (Sipola et al. 2002a). In the present study, no differences were seen in PRA values between the groups and PRA value was even lower in the peptide water group than in the control group. It is possible that the blood pressure lowering mechanism behind Ile-Pro-Pro and Val-Pro-Pro could be explained by an inhibition of other enzymes involving the renin-angiotensin-bradykinin system. Like ACE, cathepsin G, CAGE and chymase produce Ang II from Ang I (Kramkowski et al. 2006) (Figure 2). Chymase is located intracellularly, mainly in the mast cells, and has also been demonstrated in endothelial and vascular smooth muscle cells (Urata et al. 1993; Tom et al. 2003). Inhibition of chymase cannot explain the attenuation of the development of hypertension in rats, because Ang II production is dependent on ACE in rats (Kirimura et al. 2005; Miyazaki et al. 2006). However, because the relative peptide doses in humans are much smaller than in rats, and the results were still significant, chymase could play a role in the blood pressure lowering effect in humans. The minor ACE inhibition, on the other hand, could be enough to show effects in SHR but not in dTGR. Proline and valine have been shown to inhibit arginase *in vitro* (Dabir et al. 2006). According to this data, Ile-Pro-Pro and Val-Pro-Pro might theoretically act as arginase inhibitors and inhibit the production of ornithine in order to get more L-arginine for the NO production. It can therefore be hypothesised that some other enzymes besides ACE in the RAS or some other systems might also play a role in the beneficial cardiovascular effects of Ile-Pro-Pro and Val-Pro-Pro.

Kinetics

Considering that the tripeptides Ile-Pro-Pro and Val-Pro-Pro are able to lower blood pressure, it can be assumed that they must be absorbed intact from the intestine

or metabolised into the active components and then reach the target organ. It has been demonstrated that Ile-Pro-Pro and Val-Pro-Pro are absorbed from the gastrointestinal tract, but the evidence for this has been limited, and no data have been presented concerning the tissue distribution, excretion and time-dependent kinetics of these tripeptides. Masuda et al. (1996) detected the tripeptides Ile-Pro-Pro and Val-Pro-Pro in the aorta after oral administration to the rats (Masuda et al. 1996). The average dose of tripeptides in that study was approximately 0.36 mg/kg, whereas in the present study the dose of Ile-Pro-Pro was much higher, 7 mg/kg, based on the earlier studies (Sipola et al. 2001; Sipola et al. 2002a). Masuda et al. (1996) identified the absorbed tripeptides in two collected peptide fractions from the chromatographic separation of the extract of the aorta by amino acid composition, and determined that the amino acid composition could correspond with Ile-Pro-Pro and Val-Pro-Pro. Satake et al. (2002) have reported Val-Pro-Pro being transported via the Caco-2 cell monolayer by paracellular diffusion *in vitro*, although high amounts of tripeptides are also degraded into amino acids (Satake et al. 2002). In a very recent study, the absorption of Ile-Pro-Pro was evaluated by measuring the plasma concentration of the peptide before and after intake. The plasma concentration increased after the intake of Ile-Pro-Pro, and it was therefore thought that Ile-Pro-Pro was absorbed through the intestine and reached the circulation undegraded (Foltz et al. 2007). In the present study in rats, the maximal concentration of radiolabelled Ile-Pro-Pro in blood after oral administration was about one-third of that seen after intravenous administration. Oral absorption reached the maximal level after one to six hours.

The present study showed that, in rats, Ile-Pro-Pro is absorbed intact from the gastrointestinal tract after a single oral dose. Radiolabelled Ile-Pro-Pro was detected from several tissues and its excretion was slow; even after 48 hours, the radiolabelled peptide had not been completely excreted. The protein-binding properties were also evaluated, and it was shown that Ile-Pro-Pro did not bind to albumin or to other plasma proteins, whereas the dipeptide Pro-Pro was almost entirely bound to plasma proteins. These two peptides are similar in acid nature and therefore acid nature is not proposed to explain this protein binding difference. Concerning medicines, only the unbound part is pharmacologically active because the protein-bound part cannot cross biological membranes and is therefore unable to reach the target organ (Limberis et al. 2007). This supports the hypothesis that the blood pressure lowering effect is mediated via free Ile-Pro-Pro.

The kinetic study gave evidence that Ile-Pro-Pro might accumulate in tissues related to blood pressure regulation. Tissue-located RAS is present in the brain, kidneys, adrenal cortex, heart and blood vessels, and in the present study considerable radioactivity levels were found in the adrenals, aorta and kidneys.

6.4 CLINICAL RELEVANCE

Blood pressure

In the present study, the reductions in blood pressure values in the peptide group compared to the placebo group in the three clinical studies were 2.0 to 6.7 mmHg in systolic blood pressure and 1.8 to 3.6 mmHg in diastolic blood pressure. Meta-analysis of 17 randomised trials of antihypertensive treatment in the literature shows that reductions of 10–12 mmHg in systolic blood pressure and 5–6 mmHg in diastolic blood pressure reduce the incidence of stroke by about 38% (MacMahon and Rodgers 1996). A reduction of 2 mmHg in systolic blood pressure reduces the overall expected risk of stroke by about 4%, as it does the risk for myocardial infarction. With this blood pressure reduction, the cost savings in terms of treatment of stroke and myocardial infarction were also marked (Selmer et al. 2000). Furthermore, a reduction in systolic blood pressure of about 8 mmHg and in diastolic blood pressure of about 4 mmHg have been shown to reduce the incidence of dementia by 50% from about 8 to 4 cases per 1000 patient-years (Staessen et al. 2007).

Arterial stiffness

Arterial stiffness parameters such as AIx and Tr have been shown to be risk factors for cardiovascular events (Nurnberger et al. 2002; Weber et al. 2004). Patients with normal angiograms have significantly less arterial stiffness than subjects with evidence of obstructive coronary disease at angiography. Pulse wave velocity (Tr can be used as a substitute) was about 0.8 ms (9%) higher and AIx about 5.5% (23.5%) higher (Covic et al. 2005). Every 10% increase in AIx augments the risk of major adverse cardiovascular events (such as cardiovascular death, heart attack or stroke) by 28% in patients with coronary artery disease (Chirinos et al. 2005). In the present study, the change in AIx value was about -12% in the peptide group vs. the placebo group. This result can be considered clinically significant.

Safety

Peptide milk is a milk product fermented by the *Lactobacillus helveticus* bacteria, which have been generally used in cheese-making. In the present study, the tolerability of the peptide milk was evaluated in long-term use (21 weeks) and with high doses of tripeptides (10 and 24 weeks). This was done by asking the subjects whether or not they had noticed any adverse effects during the intervention period. A few adverse events were reported and they were similar in both groups. No changes in the safety markers (blood cell count, serum creatinine, urate and gamma glutamyl transferase) were observed. Similarly, adverse effects were not reported in earlier randomised controlled trials with milk products containing the tripeptides Ile-Pro-Pro and Val-Pro-Pro (Hata et al. 1996; Seppo et al. 2002; Mizushima et al. 2004; Tuomilehto et al. 2004), and in the current literature, no references concerning about the allergenicity of these tripeptides have been found. Peptide milk can therefore be considered a safe alternative for the dietary treatment of hypertension and vascular function.

7 SUMMARY AND CONCLUSIONS

The present study investigated the effects of the peptide milk containing the tripeptides Ile-Pro-Pro and Val-Pro-Pro on blood pressure and arterial stiffness in hypertensive subjects and the blood pressure lowering mechanisms behind these tripeptides and kinetics by using different experimental models. The main findings in the present study are as follows:

1. *Experimental*

- ▶ The ACE inhibitory activity of Ile-Pro-Pro and Val-Pro-Pro does not explain their blood pressure lowering effect, and it can be hypothesised that some other enzymes in the RAS might also play a role in this effect.
- ▶ Using radiolabelled Ile-Pro-Pro, the tripeptide was shown to be absorbed partly intact from the gastrointestinal tract, and considerable radioactivity levels were distributed in tissues related to the RAS.

2. *Clinical*

- ▶ The peptide milk containing the tripeptides Ile-Pro-Pro and Val-Pro-Pro reduced blood pressure in long-term use in hypertensive subjects when blood pressure was measured by using 24-hour ABPM.
- ▶ The peptide milk reduced arterial stiffness. It can be suggested that this effect of peptide milk is mediated via improved basal endothelial NO release, reduced blood pressure or arterial tone, although the maximal capacity of NO release remained unaffected.

In conclusion, the peptide milk lowered blood pressure and arterial stiffness in hypertensive subjects. The tripeptide Ile-Pro-Pro is absorbed from the intestine and might accumulate in tissues, including those related to the RAS. The precise blood pressure lowering mechanism of the peptide milk remains to be studied.

8 ACKNOWLEDGEMENTS

This study was carried out at the Institute of Biomedicine, Pharmacology, University of Helsinki and at Valio Ltd, Research and Development, Nutrition Research, Helsinki, during the years 2002–2007.

I have had the privilege to be supervised by Docent Riitta Korpela, PhD, and Professor (emeritus) Heikki Vapaatalo, MD. I wish to express my deepest gratitude to Riitta Korpela, who has encouraged me and given me the never-ending support I needed during these years. Her enthusiasm for scientific work is admirable and has also inspired me to enter the field of science. Her flexibility has also been very valuable during the final phase of this thesis. I also wish to express my warm gratitude to Heikki Vapaatalo, whose guidance, excellent advice, enthusiasm and wide knowledge of hypertension has been invaluable. He has always been willing to find some time to discuss my work and encourage me when needed.

I wish to express my respectful thanks to Professor Tiina Mattila-Sandholm, Senior Vice President of Valio R&D for supporting me during this time and making it possible to finish my thesis. I also owe my respectful thanks to Professor Esa Korpi the Head of the Department of Pharmacology.

I owe my sincere thanks to the reviewers, Professor Hannu Korhonen, MTT Agrifood Research Finland, Biotechnology and Food Research, Jokioinen and Professor Heikki Ruskoaho, MD, PhD, Department of Pharmacology and Toxicology, University of Oulu for their expertise.

I am grateful to all my co-authors for their expertise and support during this thesis. Katariina Wuolle MSc and Leena Seppo MSc I warmly thank for their invaluable help in planning and running the clinical trials. I offer my warm thanks to Outi Kerojoki MSc and Kari Nurmela MSc for their expertise and Mirkka Schaller PhD, Marika Collin PhD and Zhong Jian Cheng PhD for their expertise

and help in running the experimental trial. I had the privilege of collaborating with experienced specialists Sinikka Kyrönpalo MD, Martin Rasmussen MD, Mats Rönnbäck MD and Per-Henrik Groop MD. I had also the privilege of collaborating with Professor Eero Mervaala and his research group during these years. I also give my warmest thanks to Tuija Poussa MSc and Hannu Kautiainen BA for their invaluable help with statistical analyses and professional statistical advice.

I would like to thank all the volunteers who participated in the studies at Valio Ltd.

I also owe my special thanks to my present and past colleagues and co-workers at Valio. Katja Hatakka MSc, our Research Manager in Valio Ltd in Research and Development, for supporting me during these years and especially for the flexibility and support during the final stage of the thesis. Kajsa Kajander MSc, Riina Kekkonen MSc, Tuula Tuure PhD, Netta Lummela, Leena Seppo MSc, Anu Kosonen, Anna Lassig, Annikki Geier-Rajala, Minna Hietala, Suvi Vaaraniemi-Junnila MSc, Riikka Kasurinen MSc, Taru Pilvi MSc, Eveliina Myllyluoma MSc and Laura Piirainen MSc: thank you for the numerous innovative conversations and cheerful moments in and out of the research world. I will also give my warm thanks to Elina Lausvaara and Riitta Puttonen for their skilled laboratory work. And all my dear friends Anna, Anne, Sanna, Niina, Johanna and Teija, your support and trust have been really invaluable during these years.

I owe my warmest thanks to my family. I am really grateful to my parents Mervi and Tapio Jauhiainen, who have always supported and encouraged me in numerous ways throughout my life. I also give my special thanks to my big brother Tommi and his wife Nina, who have reminded me of the life outside of research and who have always been there for me. And to my "*varavanhemmat*" ("vice-parents") Matti and Anja Jauhiainen who have been the greatest support during these years in helping and supporting me in my everyday life. I also wish to express my gratitude to all other relatives for their support.

This thesis was supported in part by a grant from the Finnish Academy and the University of Helsinki.

Helsinki, September 2007



Tiina Jauhiainen

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